FUS Mutations in Familial Amyotrophic Lateral Sclerosis in the Netherlands

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Objectives: To assess the frequency of FUS mutations in 52 probands with familial amyotrophic lateral sclerosis (FALS) and to provide careful documentation of clinical characteristics.

Design: FUS mutation analysis was performed using capillary sequencing on all coding regions of the gene in a cohort of patients with FALS. The clinical characteristics of patients carrying FUS mutations were described in detail.

Setting: Three university hospitals in the Netherlands (referral centers for neuromuscular diseases).

Patients: Fifty-two probands from unrelated pedigrees with FALS.

Main Outcome Measure: FUS mutations.

Results: We identified 3 mutations in 4 of 52 probands. We observed 2 previously identified mutations (p.Arg521Cys and p.Arg521His) and 1 novel mutation (p.Ser462Phe). In addition, a p.Gln210His polymorphism was identified in 1 proband and 3 healthy control subjects. Phenotypic analysis demonstrated that patients may lack upper motor neuron signs, which was confirmed at autopsy, and disease survival was short (<36 months for 8 of 10 patients).

Conclusions: We discovered FUS mutations in Dutch patients with FALS and the occurrence of benign variations in the gene. Therefore, caution is warranted when interpreting results in a clinical setting. Although the phenotype associated with FUS mutations is variable, most patients predominantly demonstrate loss of lower motor neurons and have short disease survival.

Arch Neurol. 2010;67(2):224-230

FAMILIAL AMYOTROPHIC LATERAL sclerosis (FALS) is a genetically heterogeneous disorder characterized by progressive degeneration of upper and lower motor neurons. Patients present with muscle weakness, which gradually spreads throughout the body and eventually leads to death owing to respiratory failure within 3 to 4 years on average.1,2 Ten different types of FALS can be distinguished, and there is phenotypic variability among the different forms of FALS. Juvenile-onset forms with autosomal recessive inheritance of the disease exist, and patients may also experience cognitive impairment and parkinsonism.3 In approximately 10% to 20% of patients with FALS, mutations in SOD1 can be identified.4 Mutations in multiple other genes (including VAPB,5 ANG,6 and TARDBP7) cause FALS as well but appear to be less common than SOD1 mutations. Linkage to several additional loci, for which the causal mutations remain to be identified, has also been reported.8,9

Recently, mutations in a novel gene, FUS (OMIM 137070), have been identified in patients with FALS.10,11 Pathological studies10,11 demonstrate loss of upper and lower motor neurons and cytoplasmic aggregation of the mutant protein. Interestingly, FUS is functionally homologous to TARDBP, suggesting a possible common pathogenic mechanism. Also, mutations in FUS have been identified at comparable frequencies to TARDBP (4%-7% and 1%-3%, respectively).11 In this study, we screened 52 independent families with motor neuron disease for mutations in FUS.

METHODS

PATIENTS

All patients were referred to the University Medical Center Utrecht, Academic Medical Center, or Radboud University Nijmegen Medical Center, which are national referral centers for amyotrophic lateral sclerosis (ALS). All patients had their conditions diagnosed according to the El Escorial criteria for ALS.12
Patients with ALS with 1 or more affected relatives were considered to have a familial form of the disease. In this study, we included 52 probands from unrelated families with a history of motor neuron disease. We included 1 individual per family, and detailed family histories were taken to exclude the possibility of distant kinship. All families were of Dutch descent (with all 4 grandparents of the affected individual born in the Netherlands), except for 1 patient, who was from Southeast Asia. All patients tested negative for mutations in SOD1, ANG, VAPB, and TARDBP, deletions of SMN1, and CAG repeats in the androgen receptor gene.

In 45 families, index patients were diagnosed as having classic ALS. In 4 families, patients were diagnosed as having ALS, frontotemporal dementia, or both. In 3 families, all affected individuals only demonstrated lower motor neuron signs.

In addition to the 52 probands, we had access to 970 healthy control subjects with medical and family histories negative for neurologic disease, taken from a prospective, population-based study on ALS in the Netherlands (as described previously).13 This study was approved by the local ethics committee, and all participants provided informed consent.

### GENETIC ANALYSIS

Venous blood samples were drawn using 10-mL EDTA tubes, and genomic DNA was extracted from whole blood using a standard salting out procedure. Sequencing was performed on all coding regions of FUS (NM_004960.2), using a 96-capillary DNA Analyzer 3730XL and a BigDye Terminator 3.1 sequencing kit (Applied Biosystems, Foster City, California) as described previously.14 Primers used in this study were described previously,14 with the exception of exons 2, 3, and 6, which were sequenced using the following primers: exons 2 and 3F, GCGATTCTCCTGCTTCAC; exons 2 and 3R, CAGGACACAGCTCGTCTC; exon 6F, GAGGTTTCTGGTCTTGC; and exon 6R, CCTCAGACATCCCTAGACAC. For exons containing mutations, 970 neurologically healthy control subjects were sequenced to confirm ALS specificity. For exon 6, a total of 430 additional neurologically healthy controls were sequenced. All mutations were confirmed in independent experiments on genomic DNA. Sequence data were analyzed in PolyPhred.15

### PATHOLOGICAL ANALYSIS

Consent for autopsy was obtained. Sections 4 μm thick were cut from formalin-fixed and paraffin-embedded cervical, thoracic, and lumbar spinal cord. Sections were incubated with FUS antibody (1:200; catalog No. A300-293A; Bethyl Laboratories, Montgomery, Texas) without pretreatment and counterstained with hematoxylin.

### RESULTS

An overview of the genetic variation found in patients and healthy controls is provided in Table 1, and pedigrees with corresponding mutations that are likely to be pathogenic are shown in Figure 1. We identified 3 exonic missense mutations in 4 of 52 probands (8%). We detected 2 previously identified mutations, c.1561C>T (found in 2 probands from pedigrees 1 and 2) and c.1562G>A (found in 1 proband in pedigree 3), in exon 15.10,11

DNA was available from an affected sibling in pedigree 3, and subsequent sequencing demonstrated this individual to carry the same mutation. We also found 1 novel mutation. The c.1385C>T mutation in exon 13 was found in 1 proband in pedigree 4, resulting in amino acid substitution from serine to phenylalanine at position 462. These mutations were not detected in 970 healthy controls.

In addition, we found c.630G>C in exon 6, resulting in an amino acid change from glutamine to histidine at position 210 in the FUS protein. We also found this variant in 1 control in our initial group of 970 healthy controls. Further analysis of an additional 400 controls demonstrated the variant in 2 individuals, suggesting c.630G>C is a rare polymorphism rather than a pathogenic mutation.

In 1 index patient, a mutation (c.24G>C) was identified in the 3′ UTR, which was not identified in the healthy controls. In this pedigree, DNA was available from 2 other affected siblings, but they did not carry this mutation. This mutation was therefore considered unlikely to be pathogenic.

The c.1566G>A mutation in exon 15 was detected in 1 healthy control and did not lead to an amino acid substitution. An additional variant (c.41G>A) was found in the 3′ UTR in 8 healthy controls. Two known single-nucleotide polymorphisms were identified: rs1052352 and rs13331793.

### CLINICAL ANALYSIS

Ten individuals from 4 pedigrees were diagnosed as having ALS, and 1 person was diagnosed as having dementia. For 8 patients with ALS, medical records were complete; for 2 patients, not all clinical data were available. We observed slightly more male patients with ALS (64%), a median age at onset of 44.5 years (range, 29-66 years), a bulbar site onset in 2 patients, a spinal site onset in

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**Table 1. Genetic Variation Found in FUS**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Base Pair Change</th>
<th>Amino Acid Substitution</th>
<th>Patients With FALS (n=52)</th>
<th>Control Subjects (n=970)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>c.630G&gt;C</td>
<td>p.Gln210His</td>
<td>1</td>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>c.1385C&gt;T</td>
<td>p.Ser462Phe</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>c.1561C&gt;T</td>
<td>p.Arg521Gys</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>c.1562G&gt;A</td>
<td>p.Arg521His</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>c.1566G&gt;A</td>
<td>p.Arg522Arg</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3′ UTR</td>
<td>216&gt;G&gt;C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3′ UTR</td>
<td>41G&gt;A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
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Abbreviation: FALS, familial amyotrophic lateral sclerosis.

<sup>a</sup>Exon 6 is based on 1370 controls.
8 patients, and a mean survival time of 41 months (range, 12-100 months). In 5 patients, no upper motor neuron signs were observed at initial presentation or follow-up visits. None of the patients with ALS demonstrated signs of cognitive impairment.

Rapid loss of lower motor neurons was a striking feature observed in several patients, illustrated by development of severe weakness within months, decreased forced vital capacities (<70%) at initial presentation, severe weight loss, mildly elevated serum creatine kinase activity (200-1000 U/L; to convert to microkatal per liter, multiply by 0.0167), and short survival (<30 months). A summary of disease characteristics for affected individuals is provided in Table 2.

In pedigree 3, all 3 patients had symmetrical, proximal weakness of the upper limbs, followed by symmetrical, proximal weakness of the lower limbs and early respiratory failure. On initial pathological examination of the spinal cord of patient II:4 in pedigree 3, ballooned neurons, neuronophagia, and gliosis were seen. These features are regarded as characteristic pathological features of infantile-onset spinal muscular atrophy. Therefore, a diagnosis of adult-onset autosomal dominant spinal muscular atrophy was initially considered (as described previously).16

**PEDIGREE 1 (p.Arg321Cys)**

The index patient (III:1 in pedigree 1; Figure 1A) was a 36-year-old man of Southeast Asian origin who presented to our clinic with rapid weight loss (20 kg in 4 months) and progressive weakness of his right upper arm.
Within a month the weakness spread to the left upper arm and both lower arms, followed by severe dysphagia.

On neurologic examination, there were no signs of cognitive impairment. Atrophy, fasciculations, and weakness were observed in the arms and abdominal muscles. Asymmetrical, pathologically brisk reflexes were observed in the arms and legs (including Babinski sign). No sensory disturbances were detected. The patient died 17 months after disease onset as a result of respiratory failure.

His father (II:1 in pedigree 1; Figure 1A) had died at 32 years of age as a result of a disease characterized by progressive weakness and dyspnea after disease duration of 2 to 3 years, which is strongly suggestive of ALS. The patient’s grandmother (I:2 in pedigree 1; Figure 1A) on the paternal side had dementia, but no medical records were available for this individual.

**PEDIGREE 2 (p.Arg521Cys)**

The index patient (III:1 in pedigree 2) presented at the age of 29 years with weakness of his right upper leg, which had developed during 2 months. Within months, weakness spread to the proximal aspects of the leg muscles and hands. He had lost 17 kg of weight in this period.

On neurologic examination, there was severe atrophy of the muscles of the shoulders, upper arms, and proximal aspect of the legs. Weakness in the upper and lower limbs was symmetrical and more pronounced proximally. Widespread fasciculations were seen. Tendon reflexes were decreased or absent, and the plantar responses were flexor. Bulbar muscles were spared, and no sensory disturbance was detected. The patient died at the age of 40 years of respiratory failure, 12 months after symptom onset.

Patient III:2 in pedigree 3 (carrying the p.Arg521His mutation) first presented at the age of 35 years complaining of fatigue and widespread fasciculations in the arms and legs. Initially, no abnormalities were found on neurologic examination or electromyography. After approximately 3 years, he noticed weakness of the shoulders, and a symmetrical proximal paresis of the upper limbs was found on neurologic examination. The patient subsequently developed dyspnea and started using noninvasive ventilatory support overnight. The weakness was progressive and spread to the upper legs, hands, and respiratory muscles. Bulbar muscles remained unaffected, and there were no sensory abnormalities. There were no signs of upper motor neuron involvement at any time in the disease course. The patient died of respiratory failure 88 months after disease onset at the age of 42.

Patient II:4 in pedigree 3 developed symmetrical proximal weakness of the upper and lower limbs at the age of 46 years. The disease progressed to almost complete paralysis (excluding the facial muscles and distal upper limbs) within 3 years. The patient died after 3½ years as a result of respiratory failure.

**PEDIGREE 3 (p.Arg521His)**

The index patient (III:1 in pedigree 3; Figure 1A) was a 39-year-old man who presented to our clinic with proximal weakness in both arms, which had progressively developed during 2 months. Within months, weakness spread to the proximal aspects of the leg muscles and hands. He had lost 17 kg of weight in this period.

On neurologic examination, there was severe atrophy of the muscles of the shoulders, upper arms, and proximal aspect of the legs. Weakness in the upper and lower limbs was symmetrical and more pronounced proximally. Widespread fasciculations were seen. Tendon reflexes were decreased or absent, and the plantar responses were flexor. Bulbar muscles were spared, and no sensory disturbance was detected. The patient died at the age of 40 years of respiratory failure, 12 months after symptom onset.

Patient II:4 in pedigree 3 developed symmetrical proximal weakness of the upper and lower limbs at the age of 46 years. The disease progressed to almost complete paralysis (excluding the facial muscles and distal upper limbs) within 3 years. The patient died after 3½ years as a result of respiratory failure.

**PEDIGREE 4 (p.Ser462Phe)**

The index patient (III:1 in pedigree 4) presented at the age of 63 years with weakness of the left hand and difficulty climbing stairs. The patient complained of muscle...
cramps and widespread fasciculations. Neurologic examination demonstrated diffuse atrophy and weakness of all limbs. Reflexes were decreased or absent. No sensory disturbances were observed. A cousin (III:2 in pedigree 4) presented at the age of 59 years with lower and upper motor neuron signs in the limbs and died 24 months after disease onset.

PATHOLOGICAL ANALYSIS OF PEDIGREE 3

The initial postmortem examination of patient II:4 was performed in 1968. Spinal cord examination for this study was performed on the original sections. There was no degeneration of the corticospinal tracts. A considerable loss of motor neuron cell nuclei was observed in all segments. A single ballooned neuron was observed in the cervical and lumbar segments with slight chromatolysis. Neuronophagia and gliosis were clearly present. The brain was not available for postmortem examination.

At postmortem examination of patient III:1, the brain and the brainstem, including the medulla oblongata, were normal macroscopically. Light microscopy revealed no signs of neuronal breakdown in the precentral and postcentral gyri. Betz cells were present and normal. The corticospinal tracts were normal, as were the appearance and numbers of motor neuron cells in the brainstem nuclei (IX, X, XI, and XII). No gliosis was seen in the brainstem. Immunohistochemical analysis revealed no accumulation of CD68+ macrophages in precentral gyri, corticospinal tracts, or brainstem. Antineurofilament and antiubiquitin staining did not show any abnormalities. The spinal cord was not available.

Postmortem examination of patient III:2 demonstrated mild hypoxic damage throughout the cortex. No signs of degeneration were observed in the cortex, thalamus, hippocampus, mesencephalon, pons, cerebellum, cerebral peduncles, or corticospinal tract. In the primary motor cortex, there was no loss of Betz cells. Severe loss of motor neurons was observed in all segments of the spinal cord. The remaining motor neurons appeared shriveled and exhibited signs of degeneration without Bunina bodies (inclusions typical for ALS).

For FUS immunostaining, only the spinal cord of patient III:2 was available. Results of immunostaining are shown in Figure 2. In concordance with previous publications on FUS,10,11 decrease of nuclear staining and varying prominent cytoplasmic staining are noted.

COMMENT

In this study, we identified 3 exonic missense mutations in 4 of 52 index patients from families with ALS. Another amino acid change found in 1 index patient (p.Gln210His) was also detected in 3 healthy controls. This finding suggests that this mutation is not pathogenic but merely constitutes benign variation in the gene. Although DNA samples from controls were sequenced in this study and previous studies, large-scale data on natural variation occurring in this gene are not available and

Figure 2. Immunostaining for FUS in spinal cord of patient III:2. Different patterns of staining can be noted, including diffuse cytoplasmic staining with no nuclear staining (A), extensive FUS staining of a circumscribed cytoplasmic inclusion (B), cytoplasmic and dendritic staining (C), and granular and filamentous, skeinlike FUS staining (D).
will be necessary to help correctly assess whether mutations cause disease. Although it has been clearly demonstrated that mutations in \textit{FUS} cause FALS, it seems some mutations may be benign; therefore, results from mutation analysis should be interpreted with caution if used in the clinic diagnostically or when providing genetic counseling.

To date, all identified mutations in \textit{FUS} cluster either in a G-rich region containing exons 5 and 6 or in a RGG-rich region at the C-terminal end of the gene containing exons 13 through 15.\textsuperscript{10,11,17} Although the exact mechanism by which mutations in \textit{FUS} cause ALS is still unknown, it seems that these 2 domains may be involved and should be the focus of functional studies.

We identified 3 mutations in 4 probands that did not occur in the control subjects. The frequency of \textit{FUS} mutations in FALS in the Netherlands (7.6\%) is comparable to that of the previously published cohorts. In a combined cohort of index patients with FALS from the United Kingdom and Australia, 8 of 198 families (4.0\%) carried mutations.\textsuperscript{10} A study\textsuperscript{11} from the United States reported mutations in 20 of 997 families (2.0\%). In the United Kingdom and Australia, 8 of 198 families (4.0\%) carried mutations.\textsuperscript{10} A study\textsuperscript{11} from the United States reported mutations in 20 of 997 families (2.0\%).

In these 2 domains, survival was variable (also within the same pedigree), ranging from 12 to 100 months. Most patients in this study (8 of 10) survived less than 36 months from disease onset. In general, survival, site of onset, and age at onset as described in the present study seem to be comparable to data published previously.\textsuperscript{10}

In summary, we discovered \textit{FUS} mutations in Dutch patients with FALS and the occurrence of benign variation in the gene. Therefore, caution is warranted when interpreting results in a clinical setting. Although the phenotype associated with \textit{FUS} mutations is variable, most patients predominantly demonstrate loss of lower motor neurons and have short disease survival.

Accepted for Publication: August 20, 2009.

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\textbf{Financial Disclosure:} None reported.

\textit{Arch Neurol} / VOL 67 (NO. 2), FEB 2010 WWW.ARCHNEUROL.COM

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Funding/Support: This work was supported by the VSB funds, The Brain Foundation of the Netherlands, Princes Beatrix Fonds, Catharijne Stichting, H. Kersten and M. Kersten, J. R. van Dijk, and the Adessium Foundation.

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