Two German Kindreds With Familial Amyotrophic Lateral Sclerosis Due to \textit{TARDBP} Mutations

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\textbf{Background:} Abnormal neuronal inclusions composed of the transactivation response DNA-binding protein 43 (TDP-43) are characteristic neuropathologic lesions in sporadic and familial forms of amyotrophic lateral sclerosis (ALS). This makes \textit{TARDBP}, the gene encoding for TDP-43, a candidate for genetic screening in ALS.

\textbf{Objectives:} To investigate the presence and frequency of \textit{TARDBP} mutations in ALS.

\textbf{Design:} Genetic analysis.

\textbf{Setting:} Academic research.

\textbf{Participants:} One hundred thirty-four patients with sporadic ALS, 31 patients with familial non-SOD1 (OMIM 147450) ALS, and 400 healthy control subjects.

\textbf{Main Outcome Measures:} We identified 2 missense mutations (G348C and the novel N352S) in \textit{TARDBP} in 2 small kindreds with a hereditary form of ALS with early spinal onset resulting in fatal respiratory insufficiency without clinical relevant bulbar symptoms or signs of cognitive impairment.

\textbf{Results:} The mutations located in the C-terminus of TDP-43 were absent in 400 controls of white race/ethnicity. The novel identified N352S mutation is predicted to increase TDP-43 phosphorylation, while the G348C mutation might interfere with normal TDP-43 function by forming intermolecular disulfide bridges.

\textbf{Conclusions:} Mutations in \textit{TARDBP} are a rare cause of familial non-SOD1 ALS. The identification of \textit{TARDBP} mutations provides strong evidence for a direct link between TDP-43 dysfunction and neurodegeneration in ALS.

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MYOTROPHIC LATERAL SCLE-rosis (ALS) is a fatal neurodegenerative disorder leading to degeneration of upper and lower motor neurons in the brain and spinal cord. Clinical hallmarks are spasticity, brisk tendon reflexes, pyramidal signs, and progressive atrophy and weakness of the skeletal musculature. The patients die within a few years after onset, usually of respiratory failure.\textsuperscript{1} Most cases are sporadic (sALS), but about 10% are familial (fALS). About 15% to 20% of patients with autosomal dominant fALS have mutations in the superoxide dismutase gene (SOD1),\textsuperscript{2} while mutations in other genes (including senataxin [OMIM 608465],\textsuperscript{3} dynactin 1 [OMIM 601143],\textsuperscript{4} and vesicle-associated membrane protein B [OMIM 605704])\textsuperscript{5} are described as rare causes of fALS.

Recently, transactivation response DNA-binding protein 43 (TDP-43) was identified as a major disease protein in the abnormal inclusions in neurons and glial cells in sALS and fALS except fALS forms due to SOD1 mutations.\textsuperscript{6,7} The TDP-43 is a highly conserved 414–amino acid nuclear protein first cloned as a protein capable of binding to the transactive response DNA element of human immunodeficiency virus type 1 and later identified as part of a complex involved in splicing of the cystic fibrosis transmembrane conductance regulator gene.\textsuperscript{8,9} It contains 2 RNA recognition motifs and a glycine-rich C-terminal region. Described functions include involvement in transcription regulation and exon skipping and a role as scaffold for nuclear bodies through an interaction with survival motor neuron protein.\textsuperscript{10} These findings make \textit{TARDBP} (OMIM 605078), the gene on chromosome 1p36.22 encoding TDP-43, an auspicious candidate for genetic screening in ALS. While this study was in progress, 13 different mutations in \textit{TARDBP} were reported in fALS (G290A, G298S, A315T, etc.).
M337V, and A382T) and sALS (D169G, G287S, G294A, Q331K, G348C, R361S, N390D, and N390S) cases.11-14

In this study, we present genetic analysis data on TARDBP in a German cohort of 134 sALS cases and 31 index patients with non-SOD1 fALS to further define the spectrum and frequency of TARDBP mutations. Although no mutations were found in sALS, 2 heterozygous missense mutations, G348C and the novel N352S, were identified in fALS. The occurrence of TARDBP mutations in 6.5% of our cohort with non-SOD1 fALS not only underlines the direct role of TDP-43 dysfunction in the pathogenesis of ALS but also implicates that screening for TARDBP mutations should be considered in all non-SOD1 fALS cases.

METHODS

SUBJECTS

DNA samples from 134 patients with sALS (mean [SD] age at onset, 57.7 [11.9] years) and 31 index patients with non-SOD1 fALS (mean [SD] age at onset, 46.5 [13.2] years) were included in the study. All patients were neurologically examined at the Department of Neurology, University of Ulm, and were diagnosed as having probable or definite ALS according to El Escorial criteria.15 Additional family members of 2 families with identified TARDBP mutations were tested. DNA sampling and genetic analysis were approved by the local ethics committee. Written informed consent for genetic analysis was obtained from each individual. In all fALS cases, mutations in SOD1, DCNT1 (OMIM 601143), and VAPB (OMIM 60574) were excluded before their inclusion in the present study.

Control samples were obtained from the following sources: 276 control subjects from the Coriell Institute (neurologically normal white control panels [mean age, 70 years]; Camden, New Jersey); 63 clinical controls from the Alzheimer Disease Center at the University of Pennsylvania, Philadelphia (47 controls [mean age, 76 years]) or from the University of Ulm, Germany (16 controls [mean age, 49 years]), and 61 brain autopsy samples without evidence of neurodegenerative diseases from the University of Pennsylvania (41 samples [mean age, 69 years]) or from the Center for Neuropathology and Prion Research, Munich, Germany (20 samples [mean age, 71 years]).

GENETIC ANALYSIS

Genomic DNA was extracted from blood or frozen brain using standard procedures. The coding region of TARDBP, exons 2 through 5 and the first 328 nucleotides of exon 6, was amplified by polymerase chain reaction using primers from adjacent intronic or noncoding regions. Polymerase chain reaction products were sequenced using a terminator cycle sequencing kit (BigDye; Applied Biosystems, Foster City, California) and were run on a capillary sequencer (ABI3130, Applied Biosystems).

SINGLE-NUCLEOTIDE POLYMORPHISM GENOTYPING OF TARDBP VARIANTS

Four hundred control samples were analyzed for TARDBP variants NM_007375 as follows: c.1176G>T (p.G348C) and c.1189A>G (p.N352S) by a chemistry-based allelic discrimination assay with “Assay by Design” probes (TaqMan) on a sequence analyzer (model 7900) followed by software analysis.

RESULTS

The genetic analysis of 31 index patients from families with ALS led to the identification of 2 heterozygous missense mutations (G348C and N352S) in exon 6 of TARDBP in 2 small German kindreds. Clinical information on family members of both kindreds is summarized in the Table.

The mutations were absent in 400 control samples. Except for a synonymous mutation at amino acid 66 in 1 sALS case, no variants were detected in TARDBP among the other 133 sALS cases or among 36 controls in which all exons were sequenced.

FAMILY A

The G348C mutation was found in the index patient (III-1) of family A (Figure 1A and B). She initially demonstrated pareses of her right hand at the age of 55 years, which spread to proximal muscles and to the opposite arm and both lower limbs, leaving her wheelchair dependent after 2.5 years. Electromyography showed acute and chronic changes in distal and proximal muscles of upper and lower limbs. Cerebrospinal fluid and routine blood variables showed no abnormalities; brain and spine magnetic resonance (MR) imaging was normal. She died of respiratory insufficiency 3 years after disease onset. The daughter (IV-1) of the index patient is 38 years old and healthy. The proband’s mother (II-1) initially manifested progressive lower motor neuron disease (MND) at the age of 31 years. The disease was classified as multiple sclerosis, although she had only slowly progressive motor symptoms. The site of onset was the right hand. During the course of her disease, she progressed to have a gait disturbance. After 5 years, she experienced asymmetric tetraparesis, and 1 year later she was wheelchair dependent. She died after a 13-year disease course of respiratory insufficiency. Only limited information is available about the proband’s grandfather (I-1). He was wheelchair dependent during the last few years of his life and died at the age of 54 years. His wife (I-2) died early at the age of 45 years without clinical signs of MND or dementia. The proband has 2 siblings aged 67 years (III-2) and 64 years (III-3). DNA was available from the unaffected III-3 family member, who did not show the G348C mutation. DNA from other affected or unaffected older family members was unavailable. No autopsy was performed in the deceased family members.

FAMILY B

The N352S mutation was found in the index patient of family B (Figure 1C and D). This subject (III-1) showed first clinical signs at the age of 40 years with impairment of fine motor skills of the right hand. Motor and sensory nerve conduction was normal, but electromyography showed acute and chronic changes in distal and proximal muscles of upper and lower limbs. Cerebrospinal fluid and rou-
Table. Clinical Features of Families With TARDBP Mutationsa

<table>
<thead>
<tr>
<th>Subject/Sex</th>
<th>Age at Onset, y</th>
<th>Disease Duration, y</th>
<th>Site of Onset</th>
<th>Disease Course</th>
<th>Electrophysiological Tests (Age Performed, y)</th>
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</thead>
<tbody>
<tr>
<td>Family A With G348C Mutation</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>II-1/F</td>
<td>31</td>
<td>13</td>
<td>Right distal upper extremity</td>
<td>Progressive, asymmetric, flaccid tetraparesis; slow progression; arms before legs; gait disturbance; wheelchair dependent after 6 y; death from respiratory failure</td>
<td>NA</td>
</tr>
<tr>
<td>III-1/F</td>
<td>55</td>
<td>3</td>
<td>Right distal upper extremity</td>
<td>Progressive, asymmetric, flaccid tetraparesis; arms before legs; wheelchair dependent; death from respiratory failure</td>
<td>NCV, EMG, MEP (56)b</td>
</tr>
<tr>
<td>Family B With N352S Mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-1/F</td>
<td>68</td>
<td>4</td>
<td>Right distal upper extremity</td>
<td>Progressive, asymmetric, flaccid tetraparesis; arms before legs; gait disturbance, but preserved ability to walk; death from respiratory failure</td>
<td>NA</td>
</tr>
<tr>
<td>II-3/F</td>
<td>50</td>
<td>4</td>
<td>Distal upper extremity</td>
<td>Progressive, asymmetric, flaccid tetraparesis; arms before legs; wheelchair dependent; death from respiratory failure</td>
<td>NA</td>
</tr>
<tr>
<td>III-1/F</td>
<td>40</td>
<td>7c</td>
<td>Right distal upper extremity</td>
<td>Progressive, asymmetric, flaccid tetraparesis; arms before legs; wheelchair-dependent after 4 y; respiratory insufficiency; clinically stable since initiation of noninvasive ventilation</td>
<td>NCV and EMG (40), MEP (46)b</td>
</tr>
</tbody>
</table>

Abbreviations: EMG, electromyography; MEP, motor evoked potentials; NA, not applicable; NCV, nerve conduction velocity.

a No subject had bulbar involvement or cognitive impairment.

b Nerve conduction velocity demonstrated normal sensory nerve action potential amplitudes and normal sensory and motor conduction times;

EMG demonstrated positive sharp waves and fibrillations in arms and legs and elongated large motor unit potentials; and MEPs were normal to musculus tibialis anterior and musculus abductor digitii quinti.

c Alive.

PREDICTED EFFECTS OF G348C AND N352S MUTATIONS

Both amino acid exchanges affect highly conserved residues of TDP-43 (Figure 2A). Glycine at codon 348 is fully conserved in TDP-43 across the phylogenetic spectrum from Homo sapiens to Danio rerio, whereas asparagine at codon 352 is conserved across all examined mammals and Gallus gallus.

Using a software program (PolyPhen; http://coot.embl.de/PolyPhen/) to predict effects on protein structure or function, the G348C mutation was predicted to have a probable damaging function, whereas no deleterious effect was predicted for the N352S mutation (Figure 2B). A network service was used to predict putative effects on phosphorylation sites (NetPhos 2.0; http://www.cbs.dtu.dk/services/NetPhos). The N352S mutations not only introduce a new serine residue at position 352 but also lead to an increased phosphorylation prediction score for serine residues at positions 347 and 350 of TDP-43 (Figure 2B).

COMMENT

We describe 2 kindreds with a familial form of ALS with autosomal dominant inheritance due to the G348C and novel N352S missense mutations in TARDBP. These mutations were not found in 400 control samples and were not reported in more than 1000 controls of white race/ethnicity sequenced in other articles. Because DNA was unavailable from other affected family members of either kindred, we cannot definitively prove that the mu-
tations cosegregate with the disease in the families. However, as discussed herein, their critical locations and predicted functional changes, together with the family history and their absence in numerous control samples, strongly support the idea that both mutations are pathogenic.

The clinical phenotype in both families with spinal onset and predominance of lower motor neuron signs with absence of bulbar signs or evidence for cognitive impairment is in accord with previous findings reported in

TARDBP

mutation cases. So far, spinal onset is described in 77% of

TARDBP

mutation cases, lower motor neuron signs were predominant in 39%, and the absence of cognitive impairment is a consistent finding.\textsuperscript{11-14} However, this clinical phenotype does not allow separating

TARDBP

mutation cases from other forms of ALS, with similar features being reported in sALS and in SOD1 fALS.\textsuperscript{16,17}

Disease onset in our 2 kindreds is within the range of described disease onset at 30 to 83 years among other reported

TARDBP

mutation cases.\textsuperscript{11-14} However, it is notable that the G348C mutation, which has been reported recently in a single sALS case,\textsuperscript{14} leads to the earliest disease onset among all

TARDBP

mutation cases, with onset at 31 years (in the present study) and at 30 years,\textsuperscript{14} suggesting that this amino acid exchange evokes severe functional changes of TDP-43.

Except for the D169G mutation, all other

TARDBP

mutations, including the G348C and novel N352S mutations reported herein, are located in exon 6 encoding for the C-terminus of TDP-43. The importance of the glycine-rich C-terminal domain of TDP-43 in mediating its exon skipping and splicing inhibitory ability has been demonstrated and has been observed to correlate with its ability to interact with other members of the heterogeneous nuclear ribonuclear A and B protein families with well-known splicing inhibitory properties.\textsuperscript{10}

The introduction of a cysteine residue due to the G348C mutation in the C-terminal region of TDP-43 is predicted to affect protein function (Figure 2B),\textsuperscript{14} possibly by the formation of intermolecular disulfide bridges, which might interfere with protein-protein interaction or might increase the aggregation tendency of TDP-43. The most likely effect of the N352S mutation is increased TDP-43 phosphorylation. This might lead to im-

Figure 1. Pedigrees of families A and B with chromatograms of part of exon 6 of

TARDBP

showing G348C and N352S mutations in the index patients, respectively. A, Family A with the G348C mutation in

TARDBP

. B, Chromatogram of part of exon 6 of

TARDBP

showing G348C mutation in the index patient. C, Family B with the N352S mutation in

TARDBP

. D, Chromatogram of part of exon 6 of

TARDBP

showing N352S mutation in the index patient. Square indicates male; circle, female; slash, deceased; solid symbol, affected; ?, possibly affected; and arrow, index patient.
Figure 2. Predicted effects of G348C and N352S mutations. A, Sequence alignment of amino acids 340 through 360 of TDP-43 and TDP-43 homologs in Homo sapiens, Rattus norvegicus, Gallus gallus, Xenopus tropicalis, and Danio rerio. B, Effects of TARDBP mutations G348C and N352S on protein structure and function predicted using a software program (PolyPhen; http://cseb.embl.de/PolyPhen/) and on phosphorylation site prediction using a network service (NetPhos 2.0; http://www.cbs.dtu.dk/services/NetPhos). The lower the score, the more benign the substitution. The higher the score, the higher the probability for phosphorylation. Phosphorylation sites for predicted scores are boldfaced. WT indicates wild-type TDP-43.

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