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Mutations in TDP-43 link glycine-rich domain functions to amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease that affects ~2/100 000 individuals each year worldwide. Patients with ALS suffer from rapidly progressive degeneration of motor neurons ultimately leading to death. The major pathological features observed in post-mortem tissue from patients with ALS are motor neuron loss, cortical spinal tract degeneration, gliosis and cytoplasmic neuronal inclusions formed by TDP-43 or TAR DNA binding Protein with a molecular mass of 43 kDa, which are now recognized as the signature lesions of sporadic ALS. TDP-43 possesses two RNA binding domains (RBD) and a glycine-rich C terminus classifying it with other heterogeneous nuclear ribonucleoproteins known as 2XRBD-Gly proteins. A number of reports showed that a subset of patients with ALS possess mutations in the TDP-43 (*TARDBP*) gene. This further strengthens the hypotheses that gain of toxic function or loss of function in TDP-43 causes ALS. Currently, 29 different *TARDBP* missense mutations have been reported in 51 unrelated sporadic or familial ALS cases and two cases of ALS plus concomitant frontotemporal lobar degeneration with a remarkable concentration of mutations in the C-terminal glycine-rich domain of TDP-43. As these mutations will most certainly be an invaluable tool for the design and implementation of ALS animal and cell models, as well as serve as a platform for exploring the pathobiology of TDP-43, here we summarize the identified pathogenic *TARDBP* mutations and their potential impact on our understanding of the role of TDP-43 in disease.

INTRODUCTION

TDP-43 is the major pathological protein in frontotemporal lobar degeneration (FTLD) with ubiquitin positive inclusions (FTLD-U) with and without motor neuron disease (MND) and both sporadic and familial forms of amyotrophic lateral sclerosis (ALS) (2). TDP-43 pathology is now recognized as the signature lesions of all sporadic ALS and some forms of familial ALS as well as most sporadic and some familial forms of FTLD-U, which is now designated FTLD-TDP (3–6). These forms of ALS and FTLD-TDP are collectively known as TDP-43 proteinopathies (7–9). TDP-43 protein pathology is characterized primarily by cytoplasmic accumulation of TDP-43 protein into round inclusions, skeins or threads that are associated with coincident nuclear clearing of TDP-43 in neurons and glial cells, but nuclear inclusions also occur albeit less commonly (2,10,11). Biochemical fractionation of post-mortem brain tissue from FTLD-TDP and ALS patients extends the definition of pathological TDP-43 by showing a significant proportion of insoluble TDP-43 with C-terminal fragmentation, high molecular weight ubiquitinated TDP-43 smears, and hyperphosphorylation of TDP-43 (2,10). In addition to FTLD-U and ALS, other TDP-43 proteinopathies with TDP-43 pathology include Alzheimer's disease, hippocampal sclerosis, corticobasal degeneration and Huntington's disease (12–14).

The familial forms of ALS (fALS) account for ~10% of all ALS patients with mutations in the superoxide dismutase gene (SOD1) being the most common of all mutations in genes linked to ALS (15). Recent studies show that both TDP-43 and FUS (FUsed in Sarcoma) are genetically linked to ALS, thereby implying that critical functions in RNA splicing and

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metabolism are disrupted in ALS (16,17). Both proteins reside primarily in the nucleus and possess similar domain organization that is consistent with their heterogeneous nuclear ribonucleoprotein (hnRNP) classification (18–21). However, within the hnRNP family of proteins, TDP-43 is most closely related to a sub-classification of hnRNPs including hnRNP-A1 known as 2XRBD-Gly proteins (22,23). They have two RNA binding domains (RBD) followed by a C-terminal glycine-rich motif and are believed to function in a similar capacity.

FUS, TDP-43 and hnRNP-A1 have similar functional roles in RNA splicing and transcription. Much like FUS and hnRNP-A1, TDP-43 is proposed to function in aspects of transcriptional regulation through RNA/DNA and protein–protein interactions (24–30). TDP-43 also regulates alternative splicing through direct RNA binding of RBD1 to intronic uridine-guanosine repeat (UG_mU_n) and glycine-rich domain interactions with other hnRNPs (18,31,32). In this respect, TDP-43 exerts a mild enhancement in RNA splicing of the cystic fibrosis transmembrane conductance regulator through mechanisms that parallel alternative splicing mechanisms involving hnRNP-A1 (18,33). Thus, comparison of TDP-43 and hnRNP-A1 may illuminate common roles for glycine-rich domains observed in 2XRBD-Gly proteins, whereas functional similarities of TDP-43 and FUS might define common biological activities that are relevant to the broad aspects of MND.

TARDBP mutations and their relationship to ALS and FTLD-TDP

The breakthrough discovery of TDP-43 protein pathology in patients with ALS and FTLD-TDP (formerly FTDL-U) (2) was rapidly confirmed by a number of independent research groups and opened up new avenues of research that implicates TDP-43 as the key pathological component in ALS and FTLD-TDP (2,8,10). Most notable is the identification of *TARDBP* gene mutations observed in a subset of patients with ALS. The current list of *TARDBP* gene mutations includes 29 different missense mutations with all but one (D169G) residing in the C-terminal glycine-rich domain encoded by exon 6 of TDP-43 (Table 1). All missense mutations were observed in patients who were clinically diagnosed with MND including 18 fALS patients ($n = 1167$ surveyed in all studies) and 30 sporadic ALS (sALS) patients ($n = 2846$ surveyed in all studies) that were not observed in control individuals ($n = 8117$ surveyed in all studies) (34–47). All *TARDBP* mutations exhibit an autosomal dominant pattern of inheritance and appear to have an equivalent gene dosage from both the wild-type (WT) and the mutant alleles (41). A recent report identifying two patients with FTLD plus MND (FTLD-MND) in whom a *TARDBP* mutation ($n = 573$ surveyed in all studies) was found illustrates that *TARDBP* mutations are not restricted to ALS alone (47). Discovery of *TARDBP* mutations in both ALS and FTLD-TDP patients reflects a recurring theme that these diseases are linked within a broad spectrum of neurodegenerative TDP-43 proteinopathies.

The *TARDBP* gene is located on chromosome 1 (1p36.22) and contains six transcribed exons. The major protein form of TDP-43 is translated from exons 2–6 resulting in a 414 amino acid protein. Collectively, there are 70 distinct *TARDBP* point mutations or variants identified to date including 28 of the above-mentioned missense mutations, two benign missense mutations, one nonsense mutation, six synonymous mutations, seven mutations in the 5′ untranslated region (UTR), 21 intronic mutations, and five mutations in the 3′-UTR region of *TARDBP*. The majority of these mutations occur in exon 6 of *TARDBP* which encodes ~60% of the TDP-43 protein and more than 70% of the entire mRNA transcript. In light of these observations, it is clear that exon 6 and its encoded glycine-rich domain are critical components of the TDP-43 protein.

Most *TARDBP* exon 6 missense mutations occur at amino acids that are highly conserved among mammals (37,40,45), but a closer examination of glycine-rich domains from other human 2XRBD-Gly hnRNPs, like hnRNP-A1 and hnRNP-A2/B1, reveals that some of these mutations occur at positions that are evolutionarily conserved among these human paralogs. For example, TDP-43 and hnRNP-A1 share ~21% sequence identity in their respective glycine-rich domains (Fig. 1) while both show regular spacing of aromatic amino acids interspersed with conserved glycines (48). Furthermore, conserved serines in the extreme C termini are known phosphorylation target sites for hnRNP-A1 and TDP-43 (49,50). Moreover, the glycine-rich domains in hnRNP-A1 and hnRNP-A2/B1 are known to serve functional roles in protein–protein interactions, RNA binding and nucleocytoplasmic shuttling (51–55). Observations that TDP-43 also facilitates protein–protein interactions through its glycine-rich domain (32,56) provide support for the notion that common hnRNP functions, when impaired, could be involved in pathways leading to neuron dysfunction and degeneration in ALS as well as in FTLD.

The A382T *TARDBP* missense mutation is the most frequent mutation observed in both fALS and sALS patients (Table 1). Although it is possible that individuals harboring the A382T mutation may have originated from the same founder (42), another missense mutation at the same nucleotide position (G1144>C: A382P) suggests that this site may be vulnerable to a high mutation rate with pathogenic consequences leading to ALS. The sequence alignment of TDP-43 and hnRNP-A1 shows that alanine 382 is not a highly conserved amino acid; however, the second most frequent *TARDBP* mutation G348C affects a more conserved residue (Fig. 1). In fact, most glycine residues identified as being affected by missense mutations in TDP-43 are conserved in hnRNP-A1. The glycine mutations in *TARDBP* are concentrated in the most glycine dense region of the C terminus of TDP-43 (amino acids 275–310) (Fig. 1), further implying that a fundamental function of this motif is affected by these mutations which are pathogenic for ALS.

Since characteristic hallmarks of TDP-43 protein pathology include hyper-phosphorylation, ubiquitination and aggregation of both full length and C-terminal fragments of TDP-43 (2,10,11,57–59), it was suggested that specific mutations in TDP-43 may alter the phosphorylation state of TDP-43 or increase TDP-43 aggregation. Thus, it is plausible that three missense mutations, i.e. S379C, S379P and S393L, are pathogenic because they abolish phosphorylation target sites of casein kinase I (42,49), thereby implying that reduced or

Table 1. Review of TDP-43 mutations

Mutation	Frequency[a]	Clincal diagnosis	TDP-43 pathology	Reference
Missense				
D169G	1	sALS	NA	39
N267S	1	sALS	Yes	42
G287S	2	sALS[b]	NA	39,42
G290A	1	fALS	NA	38
G294A	1	sALS[c]	NA	37
G294V	2	sALS/fALS	NA	42,45
G295R	1	sALS	NA	42
G295S	4	sALS/FTLD-MND[c]	NA	42,45,47
G298S	1	fALS	Yes[d]	38
A315T	1	fALS	NA	39
Q331K	1	sALS	Yes[e]	37
S332N	1	fALS	NA	42
G335D	1	sALS	NA	42
M337V	3	fALS	Yes[e,f]	37,41,42
Q343R	1	fALS	Yes[d,g]	40
N345K	1	fALS	Yes[f]	41
G348C	6	sALS/fALS	Yes[f]	35,36,39,45
N352S	2	fALS	NA	34,35
R361S	1	sALS	Yes[f]	39
P363A	1	sALS	NA	36
Y374X	1	sALS	NA	36
S379P	1	fALS	NA	42
S379C	1	sALS	NA	42
A382T	11	sALS/fALS	Yes[f]	39,42,45
A382P	1	sALS	NA	36
I383V	1	fALS	Yes[f]	41
N390S	1	sALS	NA	39
N390D	1	sALS	Yes[f]	39
S393L	1	sALS	NA	42
Benign missense				
A90V	5	sALS/FTLD-MND/Ctrl	Yes[e]	37,39,44,47,63
D65E	1	sALS	NA	44
Benign synonymous				
S29S	1	sALS	NA	42
A66A	9	sALS/fALS/FTLD-MND	NA	35,36,41,43–46
Y214Y	1	ALS	NA	46
P225P	1	sALS	NA	44
A315A	4	sALS/FTLD-MND/Ctrl	NA	36,42,44,45
N352N	1	sALS	NA	44
Benign UTRs				
5′-UTR	7	fALS/sALS	NA	41,46
Intronic	21	fALS/sALS	NA	41,42,46
3′-UTR	5	fALS/sALS/FTLD-MND/Ctrl	NA	36,41,44,47

[a]Number of unrelated individuals with mutation.
[b]One G287S patient also possess the benign A315A on distinct alleles.
[c]Patient has reported cognitive impairment.
[d]TDP-43 pathology observed in patient brain tissue by immunohistochemistry.
[e]*In vitro* transfection of recombinant TDP-43 mutant shows CTFs.
[f]C-terminal TDP-43 fragments (CTF) extracted from patient lymophoblasts.
[g]CTFs observed in patient CNS tissue.

impaired phosphorylation of TDP-43 at these sites may play a mechanistic role in the onset or progression of ALS. Conversely, nine other pathogenic missense mutations that replace glycine-rich domain amino acids with serine or threonine would be predicted to increase the hyperphosphorylation state of TDP-43 (35,39,42). Further, the Q331K and N345K mutations could be pathogenic as a result of creating novel targets for ubiquitination, whereas the G348C and S379C mutations may be predisposed to ALS by increasing the propensity for TDP-43 to aggregate through disulfide bond formation (35). Although these proposed mutation induced changes in the biology of TDP-43 are speculative and need to be examined experimentally, they illustrate strategies for generating testable hypotheses to account for the pathogenic consequences of these and other TDP-43 mutations linked to sporadic and familial ALS.

ALS families and individuals with sALS harboring missense mutations in the *TARDBP* gene were all diagnosed with ALS in keeping with the El Escorial criteria (60,61). The majority of these patients were characterized as exhibiting ALS with spinal onset in both upper and lower motor neurons (37 of 50 patients). Bulbar muscle symptoms were less frequent at the onset of disease (13 of 50 patients); however, several ALS patients who presented with upper and lower

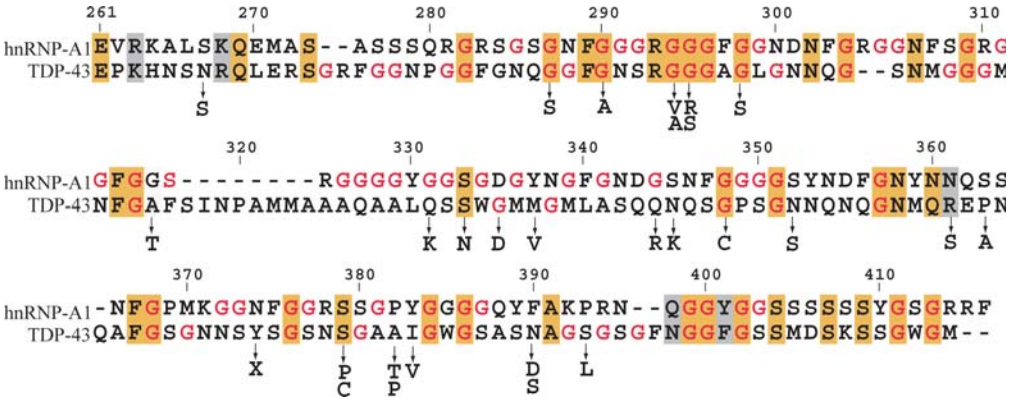

Figure 1. The glycine-rich domain protein sequence alignment of TDP-43 and hnRNP-A1 is shown with amino acid numbering (top) corresponding to the TDP-43 amino acid number. Identical and similar amino acid residues are boxed and colored in orange and grey, respectively. Glycines are highlighted in red and the arrows indicate missense mutations in TDP-43 with their corresponding amino acid change.

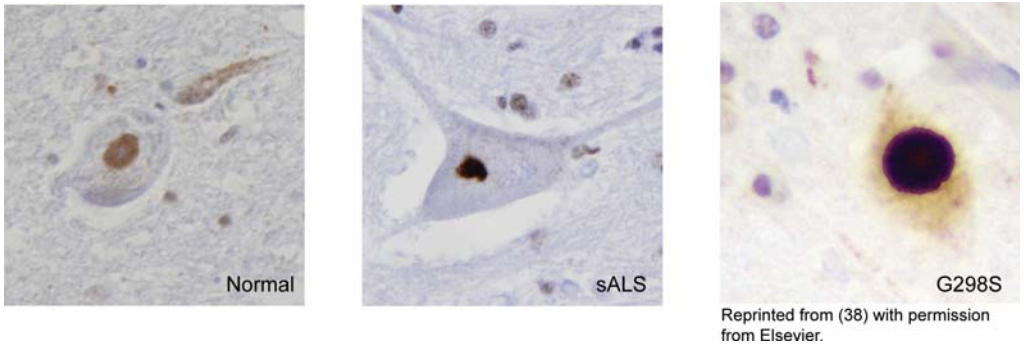

Figure 2. Immunohistochemical staining of TDP-43 in spinal cord motor neurons from control subject (left), a sporadic ALS patient (middle) and a fALS patient harboring the G298S mutation (right) illustrate that TDP-43 pathology in ALS cases with and without a *TARDBP* missense mutation is indistinguishable.

motor neuron involvement subsequently developed bulbar symptoms at follow-up examination. Further, three unrelated patients carrying *TARDBP* missense mutations were also characterized as having cognitive or behavioral impairments consistent with FTLD. Corrado *et al.* (42) noted that one fALS patient harboring the G294V mutation showed signs of cognitive impairment, whereas a study by Benajiba *et al.* (47) reported a G295S *TARDBP* missense mutation in two families with medical histories that included diagnoses of behavioral variant FTLD and semantic dementia. Observations that *TARDBP* missense mutations are found in patients with FTLD-MND and both fALS and sALS forms further support the notion that TDP-43 proteinopathies represent a continuum of neurodegenerative diseases (7).

The biochemical and pathological signatures of TDP-43 proteinopathies are present in ALS patients with *TARDBP* missense mutations. Cytoplasmic accumulations of TDP-43 accompanied by the presence of insoluble C-terminal TDP-43 fragments are hallmark pathological findings in TDP-43 proteinopathies (2,10). Immunohistochemical analysis of CNS tissue from patients with the G298S and Q343R *TARDBP* mutations clearly demonstrate the presence of skein-like, round and granular neuronal cytoplasmic inclusions as well as nuclear clearing of TDP-43 that are indistinguishable from the TDP-43 pathology in both sALS and FTLD-TDP (Fig. 2) (38,40,62). Biochemical fractionation of extracts from lymphoblastoid cell lines derived from ALS patients carrying a *TARDBP* mutation showed proteolytic fragmentation patterns that are reminiscent of the ~25 kDa C-terminal fragment observed for TDP-43 proteinopathies (Table 1) (37,39,41,42). These observations illustrate that missense mutations in *TARDBP* induce TDP-43 abnormalities in cell culture systems that clearly parallel the pathological hallmarks observed in TDP-43 proteinopathies including ALS and FTLD-TDP.

A set of benign *TARDBP* gene variants including two missense mutations (A90V and D65E), six synonymous mutations, seven mutations in the 5′-UTR, 21 intronic mutations, and five mutations in the 3′-UTR have also been identified from *TARDBP* sequencing efforts (Table 1). Although one synonymous mutation (A315A), one 3′-UTR *TARDBP* mutation and one missense mutation (A90V) were detected in normal controls, the majority of these benign *TARDBP* mutations were observed exclusively in ALS patients. Further, the only well-characterized benign variant, A90V, is potentially worth considering as a genetic risk factor for disease since the *in vitro* expression of mutant A90V TDP-43 in cell culture resulted in the partial mislocalization of this TDP-43 variant (63). The relatively high frequency of specific synonymous mutations, like A66A and A315A, reflects a surprisingly high mutation rate at specific nucleotides that are linked to disease. Further, a sALS patient with a G287S mutation in one TDP-43 allele also was reported to

harbor an A315A silent mutation in the other allele (42). Although the synonymous mutations have no apparent effect on the translated protein, it is known that other neurodegenerative MNDs such as spinal muscular atrophy stem from splicing and protein maturation defects that arise from synonymous mutations in the survival of motor neurons gene (64,65). Given the novelty of *TARDBP* mutations as a fundamental cause of ALS, the potential biological impact of seemingly benign *TARDBP* variants should not be completely ignored.

As the data summarized above continues to emerge, both loss of function and gain of toxic function models for TDP-43 proteinopathy are supported by *in vivo* and *in vitro* experiments. For example, using a chick embryo model, Sreedharan *et al*. (37) showed that TDP-43 proteins harboring a Q331K or M337V missense mutations were associated with embryonic growth defects that were not present in chicks transfected with WT TDP-43. The mechanism whereby *TARDBP* mutations affect TDP-43 functions in the chick embryo model is not clear, but the authors suggested that *TARDBP* mutations induce an apoptotic response that causes defects in chick embryo development. However, this effect may not be specific to *TARDBP* mutations since rats injected with adenovirus overexpressing human WT TDP-43 in the substantia nigra also showed evidence of toxicity that was manifested by an apoptotic loss of neurons (66). The toxicity of TDP-43 *in vivo* is supported by the analysis of TDP-43 *in vitro*, showing that it is generally prone to aggregation and that the *TARDBP* missense mutation Q331K is associated with greater tendency to aggregate (67).

In support of a loss of function model of TDP-43 mediated neurodegeneration, a fly model lacking the TDP-43 homolog protein known as TDBH exhibits motor defects that are rescued by human TDP-43 or TDBH (68). However, it is not clear whether the loss of TDP-43 function linked to MND is associated with defects in RNA splicing since RNA splicing and hnRNP interaction studies *in vitro* suggest that missense mutations in *TARDBP* do not alter these TDP-43 activities (56). However, experiments are likely underway now to determine whether *TARDBP* mutations can rescue the motor phenotype in the TDBH deletion strains. These studies will inevitably fuel further debate on the role of TDP-43 abnormalities in mechanisms of ALS and FTLD-TDP.

The general attributes of the genetic mutations identified in the initial *TARDBP* sequencing studies set the stage for understanding the critical cellular functions that are disrupted by pathological TDP-43 in MND. Indeed, the *TARDBP* mutations will certainly accelerate the pace of developing transgenic mouse models in which to study disease causing mechanisms associated with pathological TDP-43. Exon 6 and the glycine-rich domain, in particular, are a critical component of TDP-43 function. The current *in vitro* and *in vivo* data describing the biochemical and pathological characteristics of *TARDBP* mutations recapitulate features of the pathological hallmarks observed in ALS, FTLD-MND and FTLD-TDP. Given these promising advances, we anticipate that significant progress in understanding the pathological mechanisms underlying TDP-43 proteinopathies will continue to emerge from the identification and further characterization of the genetic mutations in TDP-43 linked to ALS and FTLD-MND.

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Conflict of Interest statement. None declared.

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