Altered RNA metabolism and amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in adults. Typically, patients with ALS develop progressive weakness resulting, eventually, in respiratory muscle paralysis and death in 3–5 years after the onset of the disease. No definite therapy currently exists for ALS. The biologic basis of the disease is unknown. However, ALS research has taken a dramatic turn over the last 3 years. Landmark discoveries of mutations in the transactive response DNA-binding protein (TDP-43) and fused in sarcoma/translocated in liposarcoma (FUS/TLS) as causative of ALS and demonstration that abnormal aggregation of these proteins is the proximate cause of motor neuron loss in familial and sporadic ALS have initiated a paradigm shift in understanding the pathogenic mechanism of this disease. TDP-43 and FUS/TLS are DNA/RNA-binding proteins with striking structural and functional similarities. This article reviews the current direction of research efforts toward understanding the role of RNA (ribonucleic acid) processing regulation in ALS and possible therapeutic pathways in this fatal disease.

Key Words

Amyotrophic lateral sclerosis, fused in sarcoma/translocated in liposarcoma, RNA metabolism, TDP-43

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Introduction

Amyotrophic lateral sclerosis (ALS), also called motor neuron disease (MND), is a progressive and fatal neurodegenerative disease. First described in clinical detail in the 1860s, the biological basis of ALS continues to be an enigma. No meaningful therapy currently exists for ALS. There is no hint of cure. Patients with ALS generally die 3–5 years after the onset of the disease.[1] The classical view of ALS, that it conforms to degeneration of motor neurons, including the descending corticonuclear and corticospinal tracts and their respective targets, lower motor neurons, in the brainstem and spinal cord appears simplistic. This anatomic simplicity, however, is in stark contrast to its biological diversity, reflected in part by an extensive number of genetic variants of the disease (familial ALS, fALS) that are currently recognized[2,3] [Table 1]. Only approximately 5–8% of ALS cases are familial, and less than 40% of the familial cases are explained by all currently known genetic mutations. However, an encouraging fact is that fALS clinically mirrors sporadic ALS (sALS), and understanding the molecular and cellular basis of fALS might provide major insights into the pathogenesis of sALS, eventually leading to effective therapies.

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For almost two decades, the only gene clearly associated with fALS was SOD1,[4] which accounts for 20% of fALS. The identification of SOD1 mutations[4] in 1993 led to the molecular era of ALS research, and significant insight into ALS pathogenesis has been provided through the identification of pathways directly affected by the toxicity of mutant SOD1.[1-3,5]

A major shift in our understanding of ALS pathogenesis occurred in 2006, with the identification of a 43-kDa transactive response (TAR) DNA-binding protein (TDP-43) as a key pathological substrate of cytosolic inclusions in sALS and frontotemporal lobe degeneration with ubiquitin inclusions (FTLD-U).[6] It was soon followed in 2008 by the successful discovery of dominant TDP-43 mutations as a primary cause of ALS[7,8], thus providing the proof of principle that aberrant TDP-43 can trigger neuronal degeneration and cause ALS. A total of 38 mutations have since been reported in ALS patients with or without apparent family history,[9] accounting for approximately 5% of fALS[7,8] and rare sALS[7,8] cases. The identification of TDP-43 mutations was in tandem followed by the discovery of mutations in another RNA/DNA-binding protein FUS/TLS (fused in sarcoma and

Table 1: Different types of fALS and their genetic determinants, modified from Ticozzi *et al*.[2]

ALS type	Inheritance	Chromosome	Gene	Protein/mechanism
ALS1	AD	21q22.1	*SOD1*	Cu/Zn superoxide dismutase
ALS2	AR	2q33	*Alsin2*	GEF signaling
ALS3	AD	18q21	Unknown	Unknown
ALS4	AD	9q34	*Senataxin*	RNA/DNA metabolism
ALS5	AR	15q15.1-21.1	Unknown	Unknown
ALS6	AD	16q12	*FUS/TLS*	RNA processing
ALS7	AD	20p13	Unknown	Unknown
ALS8	AD	20q13.33	*VAPB*	Vesicular trafficking
ALS9	AD	14q11	*Angiogenin*	Neurovascularization
ALS10	AD	1p36.22	*TARDBP*	RNA processing
ALS11	AD	6q21	*FIG4*	PI(3,5)P(2)5-phosphatase
ALS12	AD and AR	10p15-14	*OPN*	Optineurin
ALS-FTD1	AD	9q21-22	Unknown	Unknown
ALS-FTD2	AD	9p13.3-21.3	Unknown	Unknown

AD, autosomal dominant; AR, autosomal recessive; FIG4, a phosphatidylinisitol-3,5-biphosphate lipid signaling protein; FUS/TLS, fused in sarcoma/translocated in liposarcoma; OPN, optineurin; TARDBP, transactive DNA binding protein; VAPB, VAMP (vesicle-associated membrane protein)-associated protein B

translocated in liposarcoma) in 2009, as a primary cause of fALS.[10] In a short period of 1 year, over 30 different FUS/TLS mutations[9] have been described in fALS, accounting for another 4% of fALS[10] and rare sALS[11] cases. In TDP-43- and FUS/TLS-associated ALS, the site and age at disease onset are variable, as also observed in sALS, and incomplete penetrance has been documented for several of these mutations, which may account, at least in part, for the absence of family history in apparently sporadic patients.[9,11] Most patients with TDP-43 and FUS/TLS mutations develop classical ALS phenotype with classical pathological features.

A careful review of other rare forms of fALS reveals that mutations in several other genes that encode RNA-processing proteins, including angiogenin, senataxin and optineurin, can also give rise to similar motor neuron degenerations [Table 1]. Taken together, the emerging concept that ALS is a RNA-processing disorder is taking a tangible shape. This review summarizes the literature pertaining to recently identified TDP-43 and FUS/TLS mutations as RNA-processing errors in ALS and its implication in understanding the pathogenic mechanism of the disease and possible future therapies.

TDP-43 and FUS/TLS as Determinants of ALS

TDP-43 is a 414 amino acid protein encoded by six exons and it contains two RNA recognition motifs (RRM 1 and 2) and a C-terminal glycine-rich region.[9] Almost all ALS-associated TDP-43 mutations are dominantly inherited and located in the glycine-rich region. Some rare sequence variants lying in the non-coding regions of TDP-43 require further studies to prove their pathogenicity.[12]

Postmortem analysis of brains and spinal cord from patients with TDP-43 mutations has found a pattern of TDP-43 cellular inclusions similar to the one previously reported in sALS.[13] In normal brain, TDP-43 is chiefly localized within the nucleus; but in ALS, TDP-43 inclusions are predominantly localized in the neuronal cytoplasm,[13,14] dysmorphic neuritis[6] and in the glial cytoplasm.[13,14] This curious but mechanistically consistent finding of TDP-43 nuclear clearance and parallel cytoplasmic accumulation suggests that cellular pathogenesis may be driven, at least in part, by loss of one or more nuclear TDP-43 functions. In this context, slight granular cytoplasmic staining of TDP-43 "pre-inclusions" is recognized in the beginning, which worsens overtime, in concert with nuclear clearing, as the ALS disease progresses.[13,14]

Immunoblotting from affected ALS brain and spinal cord has shown biochemical signatures of disease, which include full-length TDP-43, hyperphosphorylated and ubiquitinated TDP-43 and their 25 kDa C-terminal fragments (CTFs)[6] in cytoplasmic inclusions and aggregates. Curiously, CTF accumulation is dominant in the brain (compared with spinal cord), and the importance of this finding is currently unclear. Further research is required to determine the relation, if any, of the accumulation of TDP-43 and its fragments and ALS clinical phenotypes.

FUS/TLS is a 526 amino acid protein encoded by 15 exons and characterized by an N-terminal domain enriched in glutamine, glycine, serine and tyrosine residues (QGSY region), a RRM, an arginine-rich region, a glycine-rich region and a C-terminal zinc finger motif.[10,11] Most pathogenic FUS/TLS mutations are clustered in the glycine-rich region (similar to TDP-43), with rare ones in the extreme C-terminal region.[9] Similar to TDP-43, the inheritance pattern in FUS/TLS mutation is dominant, except one recessive mutation (H517Q) recently reported in a family of Cape Verden origin.[15] Just like TDP-43, most FUS/TLS mutations are missense mutations, with only few exceptions.[9] Most patients develop a classical ALS phenotype, although rare cases of ALS in the young female with FUS/TLS mutation have been reported.[16]

Like TDP-43, FUS/TLS is chiefly nuclear in distribution in most cell types. Postmortem analysis of brain and spinal cord from patients with FUS/TLS mutations has shown to possess abnormal FUS/TLS-positive cytoplasmic inclusions in neurons and glial cells.[10,15,17] These mislocalized immunoreactive FUS/TLS inclusions are strikingly non-reactive for TDP-43, implying that neurodegenerative processes driven by FUS/TLS are independent of TDP-43 mislocalization. In FUS/TLS-linked ALS with abundant cytoplasmic FUS/TLS-positive inclusions, FUS/TLS nuclear immunostaining is only slightly

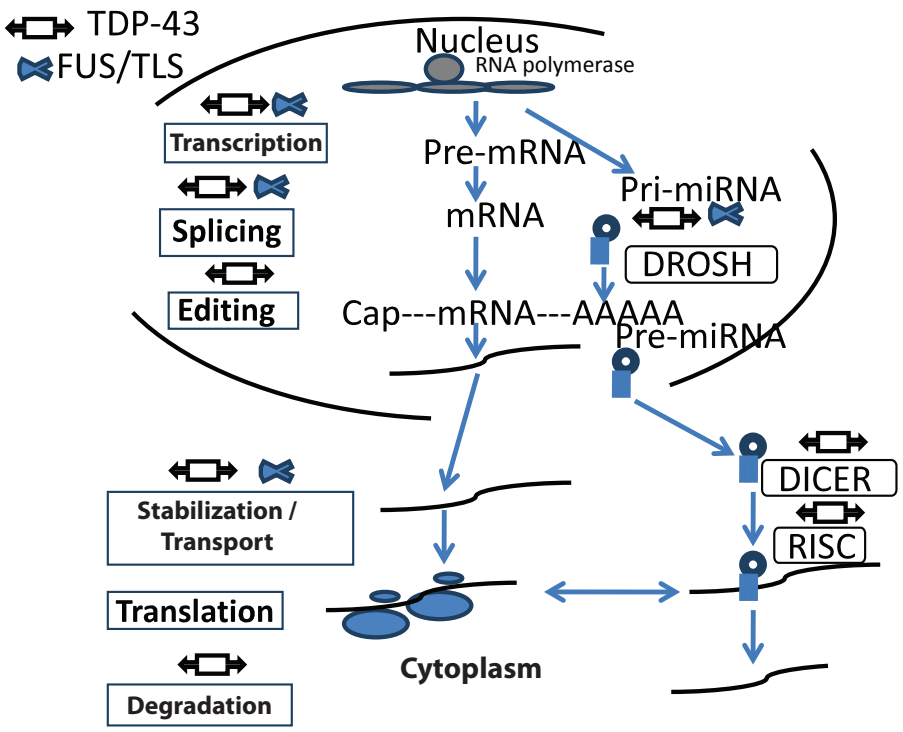

Figure 1: Schematic diagram showing steps in which transactive response DNA-binding protein-43 and fused in sarcoma/translocated in liposarcoma RNA-binding proteins are involved in RNA metabolism

reduced, and this pattern appears to be somewhat different from that in TDP-43 proteinopathy.[9,10] A further difference is that increased levels of full-length FUS/TLS protein are present in the cytoplasmic-insoluble fraction, but evidence of other biochemical abnormality such as hyperphosphorylation and ubiquitination is generally absent in FUS/TLS.[17]

TDP-43 and FUS/TLS Regulate RNA Processing

RNA processing is a tightly regulated and highly complex pathway, and it includes transcription, pre-mRNA splicing, editing, transportation, translation and degradation of RNA. Figure 1 summarizes the roles of TDP-43 and FUS/TLS in the different steps of RNA processing. TDP-43 and FUS/TLS are involved in both mRNA and miRNA processing [Figure 1].

RNA transcription regulation

TDP-43 was originally identified as a transcriptional repressor that binds to TAR DNA of the immunodeficiency virus type 1 (HIV-1), and thus its name.[18] Consistent with its role in transcription, TDP-43 was subsequently found in human brain[19] and in several cell culture systems,[20] and was shown to be associated with euchromatin – actively transcribed genes – in nuclear DNA, and its RRM2 was proposed to mediate this binding to nuclear DNA.[20]

FUS/TLS is a member of the TET (TLS, Ewing's sarcoma, TATA-binding protein-associated factor II-68) protein family that also includes the *Drosophila cabeza* protein.[3,21] FUS /TLS was initially identified as a fusion protein caused by chromosomal translocation in human cancers, and thus the name "fused in sarcoma and translocated in liposarcoma." FUS/TLS plays a major, although indirect, role in transcription by associating with general and specialized factors to influence transcription initiation.[22] FUS/TLS is also shown to interact with several nuclear hormone receptors and gene-specific transcription factors.[23] In addition, a recent study also provides a link between direct FUS/TLS RNA-binding properties and its role in transcription regulation.[20]

RNA splicing regulation

TDP-43 and FUS/TLS associate with other splicing factors in the spliceosome, and their depletion or overexpression affects the splicing pattern of specific targets. One of the best-characterized examples of alternative splicing regulation for TDP-43 comes from the cystic fibrosis transmembrane regulator (CFTR) transcripts. The pre-mRNA of CFTR contains an intronic UG track that is recognized by TDP-43, thereby skipping exon 9 in CFTR mRNA.[24] Further, it has been shown that TDP-43's major interactor protein, hnRNP A2, is a crucial component of splicing regulation, as its knockdown in cells inhibits the exclusion of CFTR exon 9.[25] TDP-43 is known to affect the splicing of apolipoprotein A-II and survival motor neuron (SMN) transcripts.[26] SMN mutation is the underlying cause of human spinal muscular atrophies, another form of MND.

Less is known about the role of FUS/TLS in RNA splicing regulation. However, proteomic analysis has identified FUS/TLS as a part of the spliceosome machinery. FUS/TLS associates *in vitro* with large transcription-splicing complexes that bind the 5′ splice site of pre-mRNA. FUS/TLS has also been shown to directly bind the pre-mRNA 3′ splice site [Figure 1], and it

has been recently shown to influence alternative splicing of the H-ras mRNA.[27]

Despite evidence that TDP-43 and FUS/TLS are involved in RNA splicing, their specific RNA targets in nerve cells have not yet been identified and a comprehensive protein–RNA interaction map still needs to be defined. The observation of a widespread mRNA splicing defect in TDP-43 and FUS/TLS proteinopathies would reinforce the crucial role of splicing regulation for neuronal integrity and search to identify candidate genes whose altered splicing is center to ALS and other related MNDs.

RNA nuclear export, cytosolic localization, translation and decay

TDP-43 and FUS/TLS have been shown by heterokaryon assays to shuttle between the nucleus and the cytoplasm[9,28-30] [Figure 1]. Indeed, both proteins are present in variable amounts in the cytoplasm, where they are involved in diverse aspects of RNA metabolism, regulating the mRNA fate in space and time, i.e. its subcellular localization, translation and degradation.

In the neuronal cytoplasm, TDP-43 and FUS/TLS are found in RNA-transporting granules that translocate to dendritic spines upon specific neuronal stimuli.[28,29] The loss of TDP-43 is known to reduce branching as well as synaptic formation in Drosophila neurons.[30] It is also shown that the cultured hippocampal neurons from FUS/TLS knockout mice[31] display abnormal neuronal spine, morphology and density.[29] Collectively, these results suggest that TDP-43 and FUS/TLS play a role in the modulation of neuronal plasticity by altering mRNA transport and local protein translation in the neurons.

RNA regulation by micro-RNA species

Although the field of micro-RNA (miRNA) is still new and just unfolding before us, early observations indicate that TDP-43 and FUS/TLS may play roles in miRNA processing [Figure 1]. miRNAs result from a two-step processing mechanism comprising of cleavage of pre-miRNA in the nucleus by the RNAase "DROSA." TDP-43 and FUS/TLS have been shown to associate with DROSA[30,31] [Figure 1], which mediates first-step miRNA processing. In addition, TDP-43 is found to be involved in the cytoplasm cleavage step of miRNA biogenesis – mediated by another RNAse "Dicer" – as evidenced by its association with proteins known to be part of the Dicer complex[30] [Figure 1]. The finally processed miRNA is an approximately 21-nucleotide-long RNA duplex that is unwound and incorporated into the "RNA-induced silencing complex" or RISC, which degrades or translationally silences mRNA. Much more insight is expected in the near future on the possible involvement of TDP-43 and FUS/TLS in miRNA processing.

Lessons Learned from Emerging Cellular and Animal Models

It is currently unclear whether loss of TDP-43 and FUS/TLS in the nucleus (loss of function) or its accumulation in cytosplasmic inclusions and aggregates (toxic gain of function), or both, lead to ALS pathogenesis. We stand to learn more from the creation of transgenic animals carrying human wild-type TDP-43 and FUS/TLS and their mutants in experimental models. Animal models for the most recently identified FUS/TLS have not yet been studied; however, for TDP-43, the initial research findings pertaining to disease modeling in Drosophila and mice have produced somewhat conflicting results. Findings from more ongoing research will likely settle the key questions concerning mutant TDP-43-mediated pathogenesis of ALS.

Wild-type and mutant TDP-43 and ALS

Expression of excess wild-type TDP-43 has been found to cause neurodegeneration in Drosophila,[32] rodent models[33,34] and in primary neuron culture systems.[35] Although these observations argue for the pathogenic role of elevated wild-type TDP-43 levels, it remains unclear whether increased levels of TDP-43 are common findings in patients with TDP-43 proteinopathies. Further, how forced synthesis of higher levels of TDP-43 relates to pathogenic mechanism in a physiologically relevant context is not established by these research approaches. Preliminary reports indicate that TDP-43 copy number[36] and brain TDP-43 mRNA levels are unchanged in human ALS.[9,36] One recent report of elevated 3′ UTR variant of TDP-43 mRNA levels[37] in human frontotemporal dementia and ALS requires replication by other groups. It is noteworthy, however, that TDP-43 mRNA levels are elevated in the Wobbler mouse model of MND.[38]

Expression of human TDP-43 carrying disease-associated mutation is found to exhibit higher toxicity (compared with wild-type) in primary rodent neurons,[35] chick embryo[7] and zebra fish.[39] Recently, accumulation of mutant TDP-43 carrying the A315T mutation in mice was reported to trigger an ALS phenotype, including gait abnormalities consistent with upper MND.[40] In another recent report, overexpression of mutant human TDP-43 in rat model led to widespread motor neuron degeneration and progressive paralysis.[41] Because these observations were made in a single transgenic line, it is difficult to exclude other mechanisms, such as integration-site artifacts, role of gene copy number or the presence of another point mutation during the induction of ALS phenotype in these experiments. But, these are all crucial points that will require independent validation.

TDP-43 intracellular localization and aggregates

TDP-43 is primarily a nuclear protein. In human ALS, early subtle cytoplasmic localization of TDP-43 in "pre-inclusion" stages is proposed to be a harbinger of neuronal demise and ALS development. Consistent with this hypothesis, progressive cytoplasmic TDP-43 localization is found beginning in the pre-symptomatic disease stages in mice overexpressing wild-type[34] as well as mutant TDP-43.[35] In various cell culture experiments, the expression of TDP-43 proteins carrying specific mutation that disrupts the nuclear localization sequence (amino acid 78–84) results in TDP-43 migration into the cytoplasm.[35] Cytoplasmic TDP-43 is shown to be toxic for rat primary cortical neurons.[35] Although overexpression of wild-type TDP-43 by itself can lead to increased cytoplasmic localization and cell death, pathogenic TDP-43 mutation is shown to increase the proportion of cytoplamic TDP-43 and acceleration of cell death.[35]

The role of phosphorylation of TDP-43 in ALS patients has been explored with the help of phosphor-specific antibodies

that strongly bind to phosphorylated cytoplasmic TDP-43, without recognizing normal intranuclear TDP-43 that is devoid of phosphorylation. The 25 kDa CTFs are highly phosphorylated and constitute a major part of intracytoplasmic TDP-43 inclusions. In transgenic mice expressing wild-type or mutant TDP-43, the appearance of 25 kDa CTFs is shown to progressively worsen as the disease progresses,[34] arguing for its pathogenic role. Extensive ubiquitination of pathogenic CTFs in cell models[41] also suggests that cellular degradation machineries such as ubiquitin–proteosome system and autophagy may be involved in removing TDP-43 aggregates.

Chicken Versus Egg

Nuclear loss versus cytoplasmic inclusions of TDP-43

The observation that the majority of inclusion-bearing cells in ALS patients display nuclei devoid of TDP-43 led to the hypothesis that some of the deleterious effects of abnormal TDP-43 metabolism may reflect a loss of TDP-43 function in the nucleus. Indeed, in transgenic mice expressing mutant human TDP-43, affected neurons displayed nuclei cleared of endogenous TDP-43.[34] Similarly, nuclear TDP-43 loss is shown to drive motor dysfunction in Drosophila.[32] The contribution of disease-causing mutations in nuclear "loss of function" mechanism is currently being explored.

Increased cytoplasmic localization of TDP-43 is associated with the formation of aggregates in the affected areas in ALS patients[13,14] and in animal models, including in Drosophila,[32] mice[34] and rats.[33] Likewise, in cell culture systems, nuclear loss and cytoplasmic localization of TDP-43 is shown to facilitate the formation of intracellular aggregates and cell dysfunction.[9,13,14]

Whether nuclear loss or cytoplasmic accumulation of TDP-43 is a major factor in ALS disease mechanism currently suffers from the "chicken and egg" ambiguity. Further, as in other neurodegenerative diseases where cytoplasmic aggregates are evident, an unresolved controversy is whether inclusions are neurotoxic or neuroprotective, the latter presumably through the sequestration of smaller toxic species of misfolded proteins.

Conclusions and future directions

The mounting evidence over the last 3 years strongly implicates TDP-43 and FUS/TLS in motor neuron degeneration in ALS through errors in multiple steps of RNA processing. Although a direct test linking loss of TDP-43 or FUS/TLS function in disease pathogenesis is still missing, given the known physiological roles of these proteins, it is easy to speculate that their loss of function may have profound effects on RNA processing with detrimental consequences for the cell. Although discovery of RNA processing errors in ALS is a monumental discovery, in order to decipher the ALS disease pathways, the essential next step should be the elucidation of the physiological roles of these proteins within normal central nervous system. The major question underlying ALS pathogenesis that needs resolution: Is ALS from TDP-43 or FUS/TLS mutation caused by the loss of normal function or gain of one or more toxic properties, or both? Moving forward, the need will also include exploitation of advances in high-throughput screening to identify normal TDP-43 and FUS/TLS RNA targets and the consequences of mutations on the processing of these targets. Improved modeling of ALS disease in animal and cell culture systems to understand the mechanism of TDP-43 and FUS/TLS-mediated ALS and to screen drugs for therapy is urgently needed for this devastating disease.

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