

Progress in Amyotrophic Lateral Sclerosis Gene Discovery

Reflecting on Classic Approaches and Leveraging Emerging Technologies

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Abstract

Amyotrophic lateral sclerosis (ALS) is the most prominent motor neuron disease in humans. Its etiology consists of progressive motor neuron degeneration resulting in a rapid decline in motor function starting in the limbs or bulbar muscles and eventually fatally impairing central organs most typically resulting in loss of respiration. Pathogenic variants in 4 main genes, *SOD1*, *TARDBP*, *FUS*, and *C9orf72*, have been well characterized as causative for more than a decade now. However, these only account for a small fraction of all ALS cases. In this review, we highlight many additional variants that appear to be causative or confer increased risk for ALS, and we reflect on the technologies that have led to these discoveries. Next, we call attention to new challenges and opportunities for ALS and suggest next steps to increase our understanding of ALS genetics. Finally, we conclude with a synopsis of gene therapy paradigms and how increased understanding of ALS genetics can lead us to developing effective treatments. Ultimately, a consolidated update of the field can provide a launching point for researchers and clinicians to improve our search for ALS-related genes, defining pathogenic mechanisms, form diagnostics, and develop therapies.

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Glossary

AD = Alzheimer disease; **ALS** = amyotrophic lateral sclerosis; **FALS** = familial ALS; **FTD** = frontotemporal dementia; **GO** = Gene Ontology; **GWAS** = genome-wide association study; **HSP** = hereditary spastic paraplegia; **MND** = motor neuron disease; **PMA** = progressive muscular atrophy; **rAAV** = recombinant adeno-associated virus; **RBP** = RNA-binding protein; **SALS** = sporadic ALS; **SMA** = spinal muscular atrophy; **WES** = whole-exome sequencing; **WGS** = whole-genome sequencing.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder. Progressive degeneration of upper and lower motor neurons in the motor cortex, brainstem, and spinal cord in ALS leads to various patterns of spasticity and flaccid limb weakness as well as dysarthria and dysphagia. Up to 50%–65% of patients with ALS may have a range of cognitive impairment, thus reflecting pathology in additional cortical regions.¹ The incidence is estimated to be 2–3 per 100,000.² About 10% of ALS cases have a first-degree relative with the disorder, which have helped uncover Mendelian causes of disease, termed familial ALS (FALS). Sporadic ALS (SALS) cases also have genetic risk factors, as evidenced by twin studies that indicate 61% heritability of SALS, yet only a fraction of these factors have actually been resolved.³ From a phenotypic standpoint, familial and SALS cases are similar in presentation with the major difference that the mean age at onset is about 10 years later for individuals with SALS relative to FALS.⁴ Males have a ~1.5-fold increased risk of developing SALS,⁵ although this increased incidence is not observed in familial cases.⁴ Early death occurs due to aspiration and respiratory failure in 2–5 years after the first recognition of symptoms,⁶ although specific clinical presentations of motor dysfunction can be quite heterogeneous.

There is not one biological test to confirm the diagnosis of ALS; the diagnosis is made clinically based on a specific phenotypic presentation to distinguish it from the broader classification of motor neuron diseases (MNDs). Classical ALS is defined as involving both upper and motor neurons. Less common variant forms are categorized along the broader diagnosis of ALS/MND. Approximately 3% of patients with ALS/MND will have isolated upper motor neuron involvement manifesting as pure spasticity over a longer duration of time and absence of other cognitive symptoms, which is termed primary lateral sclerosis.⁷ On the other end of the motor neuron spectrum, there are patients who only develop lower motor neuron dysfunction leading to weakness and amyotrophy termed progressive muscular atrophy (PMA). PMA is also rare, accounting for 3%–10% of ALS/MND.⁸ Clinical diagnosis requires precision as there are many other disease processes that mimic ALS including mechanical, infectious, neoplastic, and autoimmune that affect motor neurons and corticospinal tract function. To address the complexity in diagnosis, there have been widely accepted clinical and electrodiagnostic criteria established that derive from the definition that classical ALS involves multiple spinal regions as well as both upper and lower motor neuron involvement. To be given a diagnosis of clinically definite ALS, a

patient must have both upper and lower MNDs across 3 spinal regions or bulbar and 2 spinal regions. Alternatively, patients can be diagnosed with clinically probable ALS if criteria are satisfied by upper and lower motor neuron involvement in 2 regions. Electrodiagnostic studies can also provide evidence of lower MND even in the absence of clinically evident signs.⁹

Provided an accurate clinical diagnosis, the neuropathologic features are similar across most cases. Although the majority of ALS brains show no significant gross abnormalities, degeneration coalesces as atrophy in the anterior nerve roots of the spinal cord as well as occasional atrophy in the precentral gyrus.¹⁰ In patients who have clinical symptoms suggestive of frontotemporal dementia (FTD), this atrophy may extend to the frontal and temporal lobes. Microscopically, the degeneration of motor neurons is often observed as a reduction in neurons with associated astrogliosis in the anterior horn of the spinal cord and lower cranial motor nuclei in the brainstem as well as a more mild loss of motor neurons in primary motor cortex.¹⁰ Particularly in the cortex, some nonspecific features of neurodegeneration, such as microvacuolation or spongiosis, may also appear.¹¹

Initial Discoveries and Classical Approaches to Detect ALS Genes

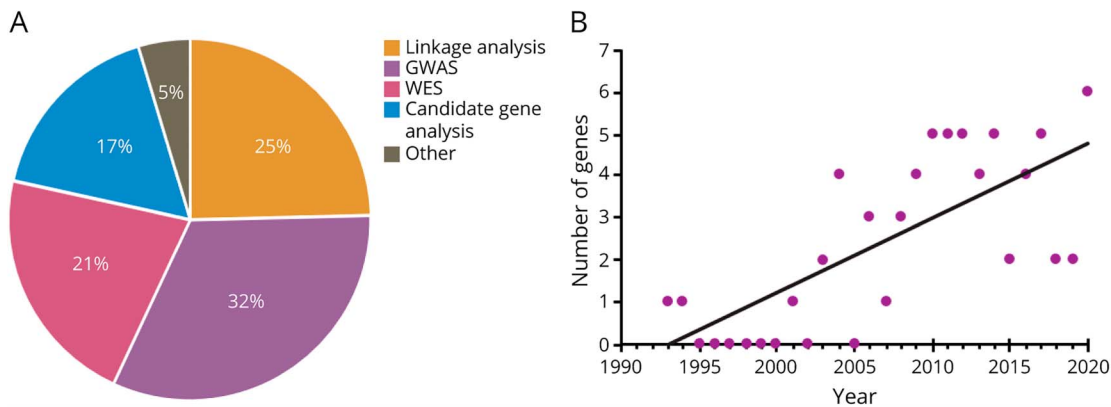
Our current toolkit for investigating genetic causes of ALS includes linkage analysis, candidate gene analysis, genome-wide association studies (GWASs), whole-exome sequencing (WES), and whole-genome sequencing (WGS). Each of these methods have individual strengths and weaknesses, and are optimized for asking specific kinds of genetic questions and have a unique use among the various discovery strategies for ALS-related genes. We highlight the kinds of studies in which each of these technologies excel as well as their weaknesses that need to be considered when designing and interpreting investigations in Table 1. Linkage studies rely on models of Mendelian inheritance and have been the most effective at identifying causal genes. Linkage analysis has accounted for about 25% of implicated genes (Figure 1A). Following these discoveries, the same genes can be identified to be associated with SALS cases using other approaches. Therefore, linkage analysis provides a robust launching point toward diagnosing ALS. With the knowledge acquired from linkage studies, the approach of candidate gene analysis can be leveraged to investigate hypotheses derived from the elucidation of associated

Table 1 Critical Look at the Strengths and Weaknesses Among Methods of Gene Discovery

Method	Strengths	Weaknesses
Classic		
Linkage analysis	• Confidence of genetic signal when sufficiently large pedigrees are used	• Few pedigrees remain unresolved
	• Segregation analysis possible	• Time consuming to collect
	• Can test for modifiers (e.g., age at onset)	• Only detects highly penetrant genes with Mendelian inheritance patterns
Candidate gene analysis	• Hypothesis driven, avoids losing statistical signal after multiple correction	• Winner's curse: results are published only for significant associations
	• Can bolster and improve proposed theories of pathogenesis	• Prior knowledge of pathogenic mechanism is necessary
		• Neglects opportunities for novel hypotheses
Genome-wide association studies	• Cost-effective, enabling analysis of large cohorts	• Does not detect causative variant (or sometimes even the causative gene) at a locus
	• Finds reproducible risk factors	• Requires precise control of diagnostic criteria and population structures (could result in spurious associations)
	• Can generate polygenic risk scores	• Prioritizes common variants (>1%), although imputation methods can be used to detect rare variants
Whole-exome sequencing	• Identifies rare variants that are causative or have a strong effect	• Only detects coding variants, thus may miss other risk factors like intronic, promoter, and enhancer variants
	• Focuses on high-priority areas of the genome (exons/coding variants)	
Whole-genome sequencing	• Captures almost all of the genome	• Short-read sequence length can miss structural variants and repeat expansions
	• Identifies noncoding contributions to disease (enhancers, promoters, and deep intronic variants)	• Currently is costly per sample
	• Can identify rare variants	
Nascent		
Integrative approaches	• Compare pathogenic variant frequency differences between different ethnic cohorts	• Positive signals may be rare and diverse and thus below statistical significance
	• Generate polygenic models and identify associations with other neurologic diseases	
	• Matches genomic data with transcriptomic and/or proteomic data to generate cause-and-effect hypotheses	
	• Detect expression quantitative trait loci (eQTLs)	
Long-read sequencing	• Can resolve structural variants and repeat expansions	• Costly and impractical for large populations
		• Harder to compare findings against databases of known nonpathogenic variants
Somatic tissue analysis	• Identify genetic variants that affect neurodegeneration even if they are not transmitted to subsequent generations, matching the sporadic nature of amyotrophic lateral sclerosis	• Need access to the various different tissue regions within the same patient
	• Elucidate regional differences in the brain	• May miss variants that have already caused degeneration of tissue
		• Difficult to ascertain how common or how significant the finding because it will often be on a by-case basis

We compare and contrast the types of studies in which classic and nascent methods are best suited for as well as important considerations of shortcomings when using any of these methods.

Figure 1 Discovery Methods and Frequency (per Year) of ALS-Associated Genes



(A) The percent contribution of various gene discovery methods to the current repertoire of 64 ALS implicated genes that we highlight. (B) The number of novel ALS gene discoveries per year. GWAS = genome-wide association study; WES = whole-exome sequencing.

pathways and genes that operate in tandem with, and similarity to, previously implicated genes. Candidate gene investigations contribute to 17% of ALS gene discoveries to date. Finally, GWAS and WES can be used more broadly to identify variants within or near genes that are associated with a risk of SALS incidence. These techniques have accounted for 32% and 21% of discoveries, respectively. Since 1993, many genes have been implicated as either causal or contributing risk, and the accelerating pace of technological advancement has led to an ever-increasing number of discoveries per year of postulated genetic contributions to ALS (Figure 1B). We highlight 46 genes with strong evidence of disease causation or association in Table 2 (along with several more putative ALS genes in eTable 1, links. lww.com/NXG/A519), and we explore the technologies contributing to their discovery. Many of these genes have been the subject of excellent recent reviews,¹²⁻¹⁶ and here, we address them in the context of technological advancement and implications for future investigations.

Linkage Studies

Classical genetics approaches including genetic linkage studies have been successful in identifying pathogenic variants in ~50%–60% of patients with a strong family history of ALS and 10%–20% of SALS.¹⁶ The first gene discovered to show linkage to familial inheritance of ALS was superoxide dismutase 1 (*SOD1*) in 1993.¹⁷ Pathologic variants are primarily missense, with a few translation termination variants appearing near the end of the protein, which account for 5%–10% of individuals with FALS. Although it was first hypothesized that *SOD1* contributed to disease by loss of the ability to eliminate reactive oxygen species, it is now generally accepted that this loss of function has a very minimal impact and that *SOD1* pathogenic variants actually lead to errors in the stability of the protein. This can cause it to fold incorrectly, not unlike prions, which can propagate misfolding among other *SOD1* proteins ultimately leading to aggregation.¹⁸

Identification of pathogenic variants in the RNA-binding protein (RBP) TDP-43, encoded by the gene *TAR DNA-binding protein* (*TARDBP*), gave us more detailed insight into ALS pathology and its relationship to RBPs and splicing regulation.¹⁹⁻²² Normally, TDP-43 is localized to the nucleus, but in patients with *TARDBP* variants and in SALS cases, it shifts to the cytoplasm and builds up as insoluble aggregates.²³ This cytoplasmic redistribution prevents TDP-43 from serving its normal function regulating mRNA splicing and leads to aberrant splicing patterns for several genes.²⁴ A heightened focus of the field to examine RBPs led to the identification of pathogenic variants in FUS RNA binding protein (*FUS*), a related heterogeneous ribonuclear protein, detected in 1%–3% of individuals with FALS.²⁵⁻²⁷ A revolutionary finding for the field informing us of an additional pathologic mechanism was pedigrees with ALS, FTD, or both found to carry hexanucleotide repeat expansions in C9orf72-SMCR8 complex subunit (*C9orf72*).^{28,29} Repeat expansions have been detected in about 40% of FALS and surprisingly 3%–7% of sporadic individuals as well.³⁰⁻³² *C9orf72* repeat expansions have also been identified in FTD, and about 15% of ALS cases also have FTD that can arise before the diagnosis of ALS.³³

These 4 genes are the most well-defined causative genes for ALS and their pathologic consequences are reflected among the molecular hallmarks of disease. All forms of ALS demonstrate ubiquitin-positive neuronal cytoplasmic inclusions in degenerating motor neurons.³⁴ With some notable exceptions, these ubiquitinated inclusions are comprised of TDP-43, p62, and other proteins. The abnormal TDP-43 inclusions observed in ALS can have varied morphology, including fine skeins, coarse skeins, dot-like inclusions, and dense round inclusions¹⁰ (Figure 2). In contrast, the motor neurons of patients with *SOD1* pathogenic variants contain abnormal ubiquitinated neuronal inclusions, but in contrast to most other forms of ALS, these ubiquitinated inclusions are comprised of accumulated *SOD1* protein and not TDP-43.³⁵ Although these TDP-43-negative/*SOD1*-positive inclusions are

Table 2 Summary of 46 Genes Associated With ALS

Gene (ref)	Method	Bearing	Pathogenic variant characteristics
ALS2 ^{e59}	Linkage analysis	Likely causative	LoF related to endosomal transport
ANG ^{e60}	GWAS	Likely causative	LoF related to angiogenesis
ANXA11 ^{e61}	WES	Likely causative	Neurotoxic cytoplasmic aggregates in neurons, defective transport
ATXN2 ^{e15}	Candidate gene	Modifier	Repeat expansion
ATXN3 ⁴¹	GWAS	Modifier	Repeat expansion
C9orf72 ^{28,29}	Linkage analysis	Definitive	Repeat expansion
CHCHD10 ^{e62}	WES	Likely causative	Mitochondrial instability, affects TDP-43
CHMP2B ^{e63}	Candidate gene	Likely causative	Impairment of autophagy
CHRNA3 ^{e64}	Candidate gene	Likely causative	Impairment of neurotransmission
DAO ^{e65}	Linkage analysis	Likely causative	LoF leading to buildup of waste products
DCTN1 ^{e66}	Linkage analysis	Likely causative	LoF, aggregates with TDP-43, impairs neuron function, synaptic instability
DNAJC7 ^{e67}	WES	High risk	Protein truncation variant
ERBB4 ^{e68}	Linkage analysis	Likely causative	Interrupts signaling, including neuregulin
EWSR1 ³⁸	Candidate gene	Likely causative	Low complexity domain, aggregation
FIG4 ^{e69}	Candidate gene	Likely causative	LoF affecting vesicular trafficking
FUS ^{25,26}	Linkage analysis	Definitive	Low complexity domain, aggregation
GLE1 ^{e70}	Candidate gene	Likely causative	Imbalance of nuclear/cytoplasmic localization, haploinsufficiency
GLT8D1 ^{e71}	WES	Likely causative	Substrate binding site, haploinsufficiency
hnRNPA1 ³⁹	Linkage analysis	Likely causative	Low-complexity domain, affects splicing
hnRNPA2B1 ³⁹	Linkage analysis	Likely causative	Low-complexity domain, affects splicing
KIF5A ⁴⁶	GWAS	Definitive	LoF affecting cargo binding
MATR3 ^{e72}	Linkage analysis	Likely causative	Can form aggregates with RBPs, interacts with splicing factors
NEFH ^{e73}	Candidate gene	Likely causative	Interferes with neurofilament cross-linking, aggregation, cell stress
NEK1 ^{e74}	WES	Modifier	LoF disrupting endosomal trafficking
NIPA1 ^{e75}	GWAS	Modifier	Poly alanine expansion, interaction with TDP-43 and C9orf72, aggregation
OPTN ^{e76}	Homozygosity mapping	Likely causative	LoF affecting NFKB, causes ER stress
PFN1 ⁴⁷	WES	Likely causative	Disrupts axonal transport
PON1,2,3 ^{e26}	Candidate gene	Risk	Gene-environment interactions leading to cell stress
PRPH ^{e77}	Candidate gene	Likely causative	Interferes with neurofilament cross-linking, aggregation, cell stress
SARM1 ^{e78}	GWAS	Risk	Unknown
SETX ^{e79}	Linkage analysis	Likely causative	Unknown
SIGMAR1 ^{e80}	Linkage analysis	Likely causative	Leads to mitochondrial injury, ER stress, and TDP-43 mislocalization
SOD1 ¹⁷	Linkage analysis	Definitive	Prion-like propagation, misfolding
SPG11 ^{e81}	WES	Likely causative	Leads to accumulation of lipids in lysosomes
SQSTM1 ^{e82}	Candidate gene	Likely causative	Impaired stress granule formation, impairs mitophagy
TAF15 ³⁷	Candidate gene	Likely causative	Low-complexity domain, aggregation
TARDBP ¹⁹⁻²¹	Linkage analysis	Definitive	Low-complexity domain, splicing misregulation, nuclear mislocalization, and cytoplasmic aggregation

Continued

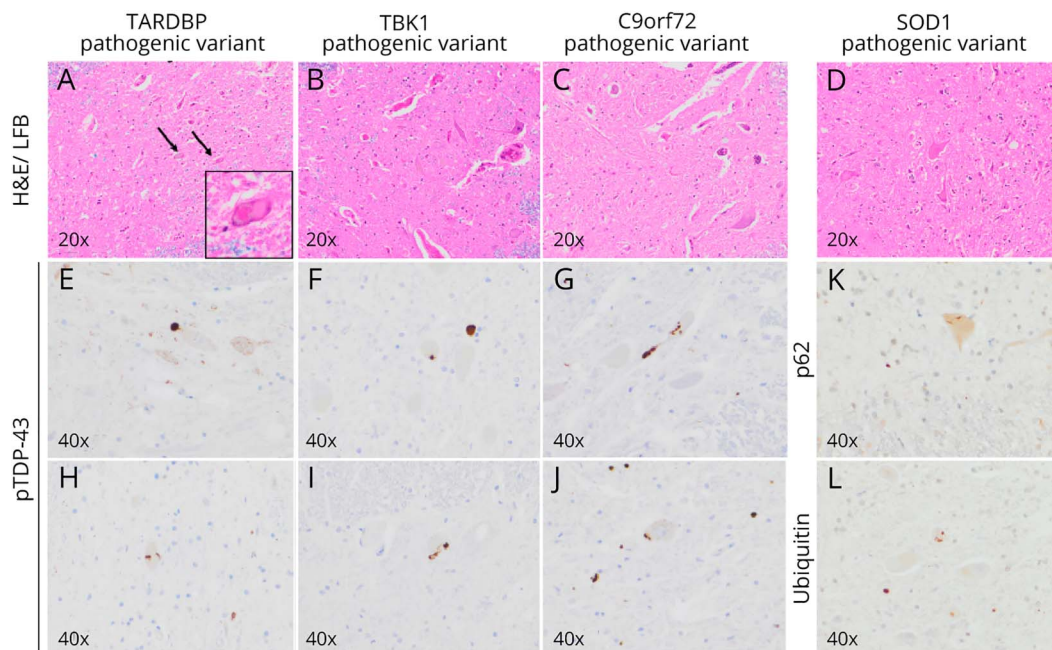
Table 2 Summary of 46 Genes Associated With ALS (*continued*)

Gene (ref)	Method	Bearing	Pathogenic variant characteristics
TBK1 ^{e74}	WES	Likely causative	LoF leading to dysfunction of phosphorylation
TIA1 ^{e83}	WES	Likely causative	Low-complexity domain, aggregation
TUBA4A ^{e84}	WES	Likely causative	Unstable microtubule polymerization
UBQLN2 ^{e85}	Linkage analysis	Likely causative	Low-complexity domain, dysfunction of UPS, and aggregation
UNC13A4 ⁴⁴	GWAS	Modifier	Intronic SNVs
VAPB ^{e86}	Linkage analysis	Likely causative	Leads to aggregation in the ER
VCP ^{e87}	WES	Likely causative	Affects aggregation and mitochondrial stress and affects FUS and TDP-43
VEGF ^{e88}	GWAS	Modifier	Promoter variant affects expression and angiogenesis and becomes burdensome during oxidative stress
WDR7 ^{e44}	Linkage analysis	Modifier	Intronic repeat expansion

Abbreviations: ALS = amyotrophic lateral sclerosis; GWAS = genome-wide association study; LoF = loss of function; SNV = single nucleotide variation; WES = whole exome sequencing. Extended details and additional genetic associations in eTable 1, links.lww.com/NXG/A519. Listed in the first column are the genes and representative references associating them with ALS. The second column names the method used for discovery. The third column notes the causal bearing of the genetic association to development of disease or whether pathogenic variants act as modifiers of disease. And finally, a brief description of the gene's pathogenic variant characteristics.

characteristic of SOD1 ALS neuropathology, abnormal SOD1 inclusions have been rarely observed in sporadic forms of ALS.³⁵ Similarly, patients with ALS related to pathogenic variants in *FUS* often demonstrate ubiquitinated neuronal

inclusions, which are comprised of mutant *FUS* protein—and not TDP-43¹⁰—although there are still cases in which the 2 still colocalize demonstrating that better characterization of molecular pathologies is still needed.³⁶

Figure 2 Common Neuropathologic Findings in ALS Subtypes

(A–D) Hematoxylin and eosin (H&E)/Luxol fast blue (LFB)-stained sections demonstrating reduction of motor neurons and astrogliosis in the anterior horn of the spinal cord. Occasionally, eosinophilic intracellular inclusions termed Bunina bodies (*TARDBP* pathogenic variant, H&E/LFB with inset) are identified. (E–J) Ubiquitinated intracellular inclusions comprised predominantly of phosphorylated TDP-43 (pTDP-43) present in patients with a *TARDBP* pathogenic variant, *TBK1* pathogenic variant, and a *C9orf72* repeat expansion. These inclusions are morphologically heterogeneous and can take the form of coarse or fine skeins, dot-like inclusions, or dense round inclusions. (K–L) Immunohistochemistry demonstrating inclusions that are positive for p62 and ubiquitin in individuals with *SOD1* pathogenic variants that have similar morphologies to the TDP-43 inclusions of the aforementioned genetic subtypes but are themselves negative for pTDP-43 immunoreactivity. Listed is the magnification in each panel. ALS = amyotrophic lateral sclerosis.

Case/Control Analysis of Candidate Genes

Candidate genes can be hypothesized to be involved in ALS based on their similarity with previously discovered genes or pathogenic mechanisms. An example of a candidate gene approach is a study that was initiated by a yeast functional screen of RBPs to find proteins that show the most similar aggregation pathology as TDP-43. Subsequently, the genes TATA-box binding protein associated factor 15 (*TAF15*) and EWS RNA binding protein 1 (*EWSR1*) were selected to be sequenced among many cases and controls, which identified variants associated with disease.^{37,38} Similarly, the identification of *TARDBP* and *FUS* ignited an interest to focus on RBPs among other familial cases. Most notable is the linkage of heterogeneous nuclear ribonucleoproteins A1 and A2b1 (*HNRNPA1*) and (*HNRNPA2b1*) within autosomal dominant families in which investigators specifically narrowed their genetic search to proteins harboring prion-like domains.³⁹ However, there is an important consideration for candidate gene approaches. The method excels at bolstering prior hypotheses of pathogenesis, yet as a consequence, it discriminates against findings that may refute these hypotheses and neglects the development of novel hypotheses.

Genome-Wide Association Studies

ALS researchers contributed to some of the early successful GWAS findings. These studies point to several significant loci including at *ACSL5*, *C21orf2*, *DPP6*, *GPX3-TNIP1*, *MOBP*, *SCFD1*, *SARM1*, *UNC13A*, and others.⁴⁰⁻⁴⁵ Many GWAS investigations have been able to identify several genes per study owing to robust data acquisition, and this has led the way in uncovering variants associated with sporadic cases.¹⁴ Furthermore, subsequent studies that screen new populations frequently replicate previous findings including those found from linkage analysis and candidate gene analysis. Obtaining convincing genetic findings from GWASs will always be a function of the sample size, population structure and composition, and the genetic effect of individual loci. More recent GWAS investigations leverage novel algorithms and imputation methods to improve sensitivity.¹⁴

The drawback of GWAS's broad approach is that significant gene variant associations can only be considered risk alleles, and subsequent studies will need to delve further into identifying actual causal variants and their contribution to disease pathology. Despite this, GWAS design is improving and still remains a practical technique leading to some of the most recent findings. Another improvement that has yet to be thoroughly incorporated is more stringent selection of phenotypes. Given the heterogeneity of ALS pathology, more careful consideration of specific phenotypes may generate novel signals.

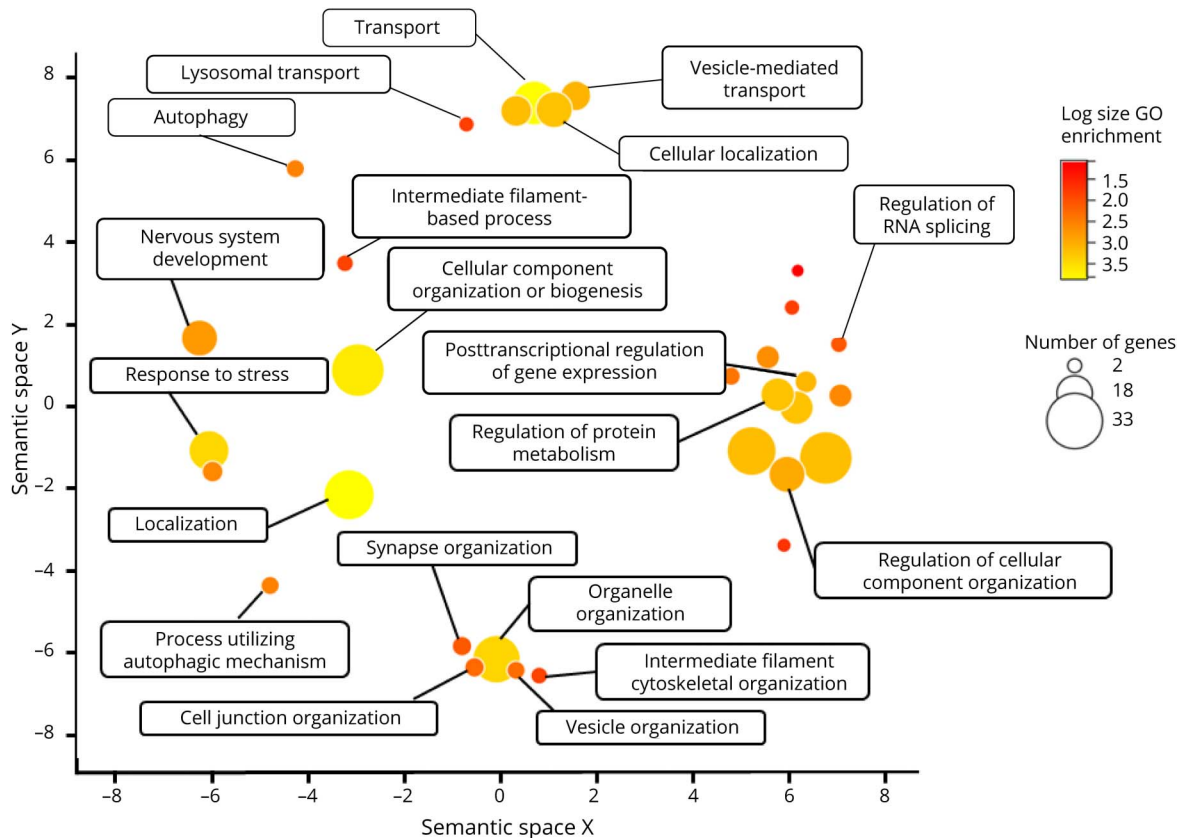
Whole-Exome Sequencing

WES takes an approach that attempts to identify an enrichment of coding variants that are more likely to be causal as opposed to GWAS, which is mostly only capable of identifying risk genes. WES can still be used in conjunction with the latest GWAS investigations to home-in on the relevance of significant GWAS signals. Using large data sets, researchers can identify variants enriched among patients with ALS. WES following a significant GWAS hit identified pathogenic variants in kinesin family member 5A (*KIF5A*).⁴⁶ The identification of *KIF5A* variants clustering at the C-terminal cargo-binding domain in ALS is particularly compelling because pathogenic variants in other regions of the gene including the N-terminal motor domain cause hereditary spastic paraplegia (HSP). Although both ALS and HSP are motor neuron disorders, the phenotypes are quite distinct. HSP arises from a dying back of upper motor neurons and typically has an earlier age at onset with longer disease duration. One proposed model for how 2 similar, but distinct, disorders can have variants in the same gene is that in HSP, the pathogenic changes broadly restrict anterograde cargo transport, whereas in ALS, they act on specific cargo proteins. WES can also be used to elucidate genes in ALS that have failed to be identified by linkage analysis as with *PFN1*.⁴⁷ In many cases, unaffected individuals also have nonsynonymous variants in genes nominated by WES approaches, and it is the increased burden of variants that can be damaging to protein function in ALS cases that ultimately contributes to disease. As more samples undergo WES (and WGS), a better resolution of the critical regions of genes and their functional implications can be established.

Whole-Genome Sequencing

The rapid decline in sequencing costs has enabled WGS technology to supplant WES. WGS techniques bypass some of the challenges of WES including probe coverage and can provide a comprehensive profile of variants across the genome. As many genes with coding variants have presumably already been identified in ALS, WGS represents an additional layer of detail to examine some of the noncoding contributors to disease such as those that may lie in promoters, enhancers, or introns. One such strategy is to look for accumulations of variants in bins across the genome.⁴⁸ The same strategy can be used near ALS candidate genes to identify regions far from coding exons. For example, using linkage analysis coupled with RNA analysis, we identified a deep intronic variant in *SOD1*, which led to the inclusion of a cryptic exon in intron 4.⁴⁹ Variants at or around the cryptic *SOD1* exon can now be identified with WGS techniques, along with other sites at genes critical for development of ALS. Despite the vast capability of WGS to scan almost the entire content of the genome, there are important technical limitations. For instance, WGS has limited capacity in

Figure 4 Revigo Gene Ontology (GO) Summary Based on the GO Panther Biological Processes Complete Statistical Overrepresentation Test^{e57,e58}



Axes reflect 2D semantic space similarity between GO terms. Color represents the number and variety of lower-level GO terms encompassed by the broader category. Size of each point represents the total number of ALS-implicated genes included in the GO category.

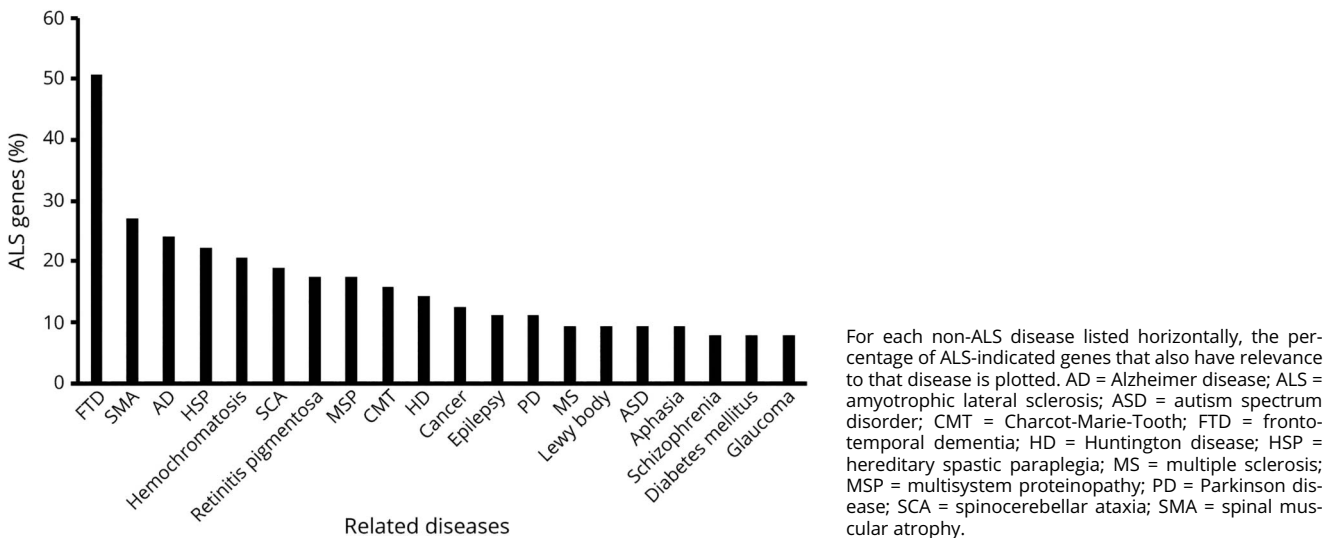
Consolidating Our Knowledge of ALS Genetics to Generate Theories of Pathology

Based on the genes identified for ALS so far, we can draw important conclusions regarding common pathways implicated and convergence toward ALS phenotypes and/or related diseases. The finding that TDP-43 mislocalization to cytoplasmic aggregates present in the overwhelming majority of patients with ALS⁵⁰ indicates a central pathology from which we can work backward to understand genetic and environmental triggers to disease progression. One step we can take toward understanding ALS is to take a holistic view of how the vast array of ALS-indicated genes is related to one another. One strategy is to construct network clusters based on the confidence of experimental evidence of functional associations using resources such as STRING (Figure 3).⁵¹ A second strategy can leverage our understanding of these genes' ontological roles to hypothesize additional genetic candidates involved in these central processes that can be screened for among patients (Figure 4).

Based on the vast amount of functional evidence gathered from investigating the effects of genetic variants in model systems, a few general understandings and theories of pathology have been popularized.⁵² Most of the genes involved can be segregated into a few overarching categories: RNA binding, proteostasis, cytoskeletal, and metabolism/redox (eTable 1, links.lww.com/NXG/A519). Ontologically, there is an enrichment of more diverse cellular processes including stress response, endomembrane system organization, localization, and RNA splicing regulation (Figure 4). An intriguing observation is that the most confident and interconnected cluster among our network of genes (highlighted in pink) is most strongly enriched for Gene Ontology terms related to RNA splicing (Figure 3). This supports theories of pathogenesis that centralize on one of the main consequences of TDP-43 mislocalization being aberrant splicing of many target genes. Of course, caution must be taken when making these interpretations because they could simply be a result of a greater investigative depth of these genes among the scientific community.

Harking back to attempting to understanding why TDP-43 and RNA regulation is a central pathology, many studies have

Figure 5 Percentage of the Listed ALS-Indicated Genes Associated With Other Diseases



shown molecular similarities among the proteins involved and their pathogenic characteristics (Table 1). An essential component of RNA regulation relies on the mechanisms by which RNA molecules interact with proteins. Highly active and ubiquitous RBPs often rely on a condensation mechanism like liquid-liquid phase separation mediated by low-complexity domains.⁵³ This mechanism must be dynamic and versatile. Because RNA regulation is spread among many proteins, any disruption to this fine-tuned mechanism can create bottlenecks in global homeostasis that are chronically exacerbated. Given that other critical functions are wrapped into this interaction network, this leads to additional imbalances in splicing regulation, nuclear export, and cytoskeletal trafficking, as well as microRNA expression. In the case of non-RBPs, pathologic aggregation can attract components of the proteostasis network and reduce its overall efficiency. RNA aggregates, including tandem repeats, can also misappropriately sequester RBPs from performing their proper function or synthesize peptide repeats that also interfere with proteostasis machinery. Overall, motor neurons, among other affected cell types, are extremely sensitive to disruptions in cellular homeostasis that chronically accumulate beyond cells' capacity to manage.⁵⁴

Challenges in ALS Genetics and Exploring Emerging Solutions

Larger Data Sets, Integrated Approaches, and Novel Computational Strategies

We have witnessed a recent push to have central repositories with an organized structure of hundreds or thousands of samples. What is becoming apparent is that single gene identifications may become increasingly infrequent, and a combination of genetic factors likely contributes to ALS

susceptibility. Project MinE is at the forefront of these approaches, with efforts to sequence the genome and connect findings with transcriptome, epigenome, and noncoding genome findings.^{55,56} Other data sets are also taking integrative approaches and providing resources for researchers at large for straightforward access to sample data. The Answer ALS consortium is one such approach, which provides whole genome data along with induced pluripotent stem cell lines and their corresponding transcriptome data, thereby saving researchers precious time and resources in trying to generate their own version of these lines. Collectively, these powerful resources will propel novel gene discoveries, especially in combination with state-of-the-art genomic methods and technologies.

Using integrative approaches that link TDP-43 binding sites with transcriptional changes has helped uncover places where loss or cytoplasmic redistribution of TDP-43 leads to improper activation of cryptic exons, for example, in the middle of intron 1 of stathmin 2 (*STMN2*).^{57,58} This activation leads to a prematurely truncated *STMN2* protein product, which is detrimental to neuronal outgrowth. More recently, another aberrant splice product creating a cryptic exon was identified in *unc-13* homolog A (*UNC13A*).⁵⁹ Intriguingly, *UNC13A* is a well-established GWAS hit for ALS,⁴⁴ and one of the lead single nucleotide variations (formerly single nucleotide polymorphisms) at this locus is directly in the cryptic exon that is generated.

With larger and more complex genomics data becoming available, the ability to process the data by individual researchers is becoming increasingly challenging. Novel machine learning and artificial intelligence applications are helping resolve some of the complexity of data analysis revealing connections between genetic variants that are not immediately obvious. These strategies have been used successfully for

imaging analysis⁶⁰ and are a promising direction in the context of ALS^{e1}. Recently, novel computational approaches have attempted to incorporate protein interactions and pathway data into machine learning models^{e2}. An accurate ALS diagnosis is important when curating increasingly larger collections of cases as there is considerable overlap between genetic associations of ALS and associations with other related disorders (Figure 5).

Ethnic Differences

Identifying genetic risk factors in multiethnic populations is currently a priority for researchers and represents an emerging yet still understudied feature of ALS.^{41,42,e3-e5} This research is important because additional genetic risk factors can be identified leading to potential breakthroughs in our understanding of ALS pathobiology. For example, the frequency of pathogenic variants in ALS-causing genes is considerably different in China compared with Europe and North America, with a less prominent role for *C9orf72* repeat expansions.^{e6}

Using samples from diverse ethnic groups has been successful at identifying or independently validating certain risk factors for African American individuals, who have a greater prevalence of Alzheimer disease (AD).^{e7,e8} Not only are some of the GWAS hits different in AD,^{e9} but relative risk at known loci for AD can be dramatically different, such as increased risk for ATP binding cassette subfamily A member 7 (*ABCA7*) variants in African Americans^{e10} compared with in non-Hispanic Whites.^{e11,e12} Examining local ancestry around a GWAS hit can also reveal that variants that have a stronger effect in certain populations.^{e13}

Oligogenic (or Polygenic) Model

Two or more sequence variants that on their own have a low probability of causing disease may have an additive or synergistic role in tandem, as has been identified in a proportion (1%–4%) of FALS and SALS cases.^{e14} Some modifying genes may have no bearing on disease susceptibility but could influence the age at onset and progression of ALS. An example is the ataxin 2 gene (*ATXN2*),^{e15} where intermediate CAG repeat expansions numbering 27–33 (but not >34, which causes spinocerebellar ataxia 2)^{e16} are enriched in patients with ALS.^{e17} Modifier genes can also explain how genes that are ubiquitously expressed (as in the case with *SOD1*, which also accounts for as much as 1% of brain protein^{e18}) proceed to elicit only a motor neuron-specific phenotype. Despite several risk genes having been reported from GWAS, there is still a lack of understanding about the functional significance of these variants. This presents an opportunity for RNA sequencing and use of disease models to interpret the potential contributions of these variants. Computational approaches have attempted to elucidate polygenic explanations and epistatic mechanisms. However, such approaches are computationally demanding, and the resources are not yet available to make groundbreaking discoveries.^{e1}

Gene and Environment Interactions

Several environmental risk factors have been proposed and interrogated for ALS. Many of these candidates originated due to increased prevalence of ALS in a particular occupation or geographical region. An unusual cluster of ALS cases was observed in Guam,^{e19} and although several hypotheses have been proposed regarding the specific neurotoxic agent, especially L-beta-N-methylamino-L-alanine, the connection is still a subject of debate.^{e20} Increased clusters of ALS have also been observed in the Kii peninsula of Japan.^{e21} More recently, false morel mushrooms (*Gyromitra gigas*) have been proposed as a risk factor for a cluster of ALS cases in the French Alps.^{e22} Military veterans are also at increased risk for developing ALS, especially those that served in the Middle East, perhaps due to repeated head trauma or exposure to neurotoxins.^{e23-e25} A unique discovery in this regard is risk variants in paraoxonases 1, 2, and 3 appeared to render soldiers more vulnerable to toxins that would trigger ALS pathology often associated with Gulf War syndrome.^{e26} Similarly, paraoxonase variants have also been connected to ALS and other neurologic disease in populations frequently exposed to pesticides.^{e27,e28} It is likely that variants in additional candidate genes predispose individuals to toxic damage on exposure to chemical agents. Traumatic brain injury among veterans and sports players may also increase the probability of developing ALS or modify the severity of disease in addition to genetic causes.^{e29,e30} And there is evidence that sterile alpha and TIR motif containing 1 (*SARM1*) deletion modifies axon injury response.^{e31,e32}

Mendelian randomization models are a powerful emerging tool that enable the discovery of causal genetics and environmental risk factors through use of summary statistics data resulting from GWASs. Although results have been mixed, there are suggestions that ALS susceptibility can be linked to type 2 diabetes, smoking, cholesterol, and physical activity.^{e33,e34} Improvement in phenotypic measurements and controlling for possible confounding variables could make this approach more practical.

Somatic Tissue Analysis for Pathogenic Variants and Repeat Expansions

Work in brain samples from individuals with autism has revealed somatic mutation events in single cells that may have been challenging to detect using bulk tissue^{e35} as well as somatic variants from high-density (~×250) coverage DNA sequencing.^{e36} A similar scenario may exist for ALS, although one of the challenges of single-cell studies is that if a cell with an offending variant dies and is cleared, the variant will not be picked up from genomic analyses. It is notable that in the same individual, *C9orf72* expansion carriers can have a much longer repeat length in the frontal cortex vs cerebellum.^{e37} This is in line with other repeat disorders, where expansions

are unique to the affected brain region, such as huntingtin (*HTT*) somatic mutations in the striatum.^{e38} It follows that sensitive and mutation-prone loci may precipitate pathology by somatic mutation events, which could explain a failure to identify a single genetic cause in some patients.

Long-Read Sequencing for Repeat Expansions and Structural Changes

A substantial fraction of the missing genetic contribution to ALS may come from tandem repeat expansions, 40 of which have been found to cause neurodegenerative diseases.^{e39,e40} Our ability to identify such repeats is limited from the technical limitations of WES and WGS. The advent of long-read sequencing technology represents an emerging strategy to uncover the contribution of repeat expansions to ALS. Increasing examples of repeat expansions with a much larger internal sequence length (in contrast to short repeats such as the *C9orf72* expansion) associated with disease are being found to have associations with AD and schizophrenia/bipolar disorder.^{e41-e43} Recently, expansions of a much larger 69-nt repeat in an intron of WD repeat domain 7 (*WDR7*) were found to be enriched in length in several ALS cohorts using long-read sequencing and length estimates from WGS data.^{e44} Repeat expansions such as those *ATXN2* and *WDR7* commonly appear insufficient on their own to cause disease, but the prevalence of their expansions in the general population is much higher than the number of individuals with ALS. Therefore, these repeats should be treated as potential modifiers of ALS or risk factors similar to how we interpret GWAS hits. In the case of *WDR7*, the expansion event can be coupled with a *FUS* variant to elicit disease.^{e44}

Copy number variants have also been explored in the context of ALS, and some examples have been identified.^{e45-e47} By performing long-read sequencing, the relative frequency of these changes can be compared with background population rates, and we can detect whether there are certain chromosomal regions and breakpoints enriched in ALS. Moreover, the approach will help detect other tandem repeats throughout the genome in an unbiased manner to reveal their relative contribution to ALS.

Conclusions

Like many neurodegenerative disorders, understanding the contributions of the many genes that are implicated in ALS remains a challenge. Classical techniques of gene discovery remain effective, and although no single approach can capture a complete picture of ALS genetics, each has strengths and weaknesses and geared toward answering specific genetics questions. The tremendous breadth of genetic, clinical, and neuropathologic information acquired thus far have aided the field in proposing a more complete model of disease. Using the most recent knowledge of common pathways can guide more refined hypotheses and experimental designs to identify

additional candidate genes either causative on their own or subtle modifiers and risk loci that increase or decrease predisposition.

One of the ultimate objectives in uncovering the genetics of ALS is to develop accurate gene therapy solutions to correct pathogenic variants. The gene therapy field is accelerating, and recent successes give hope that we can leverage our understanding of ALS genetics to develop effective treatments. The Food and Drug Administration approval of recombinant adeno-associated virus (rAAV) delivery of retinoid isomerase RPE65 (*RPE65*) for the treatment of retinal degeneration and rAAV survival of motor neuron 1, telomeric (*SMN1*) for spinal muscular atrophy^{e48,e49} are model examples. The approval was the culmination of decades of research into designing a safe and potent delivery vector. Progress in bioengineering and isolation of capsids with enhanced properties or tropism, including the ability to cross the blood-brain barrier and transduce neurons, afford the possibility of targeted delivery to specific neuronal subtypes and enhancing delivery throughout the nervous system.^{e50-e53}

Examples of promising gene therapy solutions for ALS include the delivery of antisense molecules targeting *SOD1* and *C9orf72*, which now are progressing to clinical trials.^{e54-e56} The advantage of using genes with known pathogenic variants is that therapies conceivably can be commenced before symptom onset, as is often the case for rAAV-*SMN1* delivery. This has the advantage of restoring motor neuron function before decline. Although therapies targeting familial inherited pathogenic variants are arguably the most promising for ALS, they only apply to a subset of patients, which minimizes their utility for treating the greater majority of ALS cases. However, given that up to 97% of individuals with ALS are positive for TDP-43 inclusions,⁵⁰ there may be an opportunity for gene therapy targeting TDP-43 to restore functionality, which could benefit many more patients.

An accurate diagnosis in the clinic and neuropathologic characterization are required for continuing success at identifying novel and rare genetic associations. So far, our collection of ALS genetic associations point to TDP-43 and RNA regulation as one of the most likely convergent mechanisms. However, it remains to be seen how widespread and how strong of a bearing on disease alternative pathologic mechanisms may have such as gene-environmental interactions, noncoding genetic modifiers of disease, and somatic variants. Currently, techniques better suited to finding subtle and elusive contributors to disease should be brought to the forefront. Integrative analyses, somatic mutation analysis, and long-read sequencing technologies are some of the most promising new approaches for novel gene discovery beyond the scope of protein-coding variants. Computational approaches toward identifying mechanisms of genetic epistasis and

pleiotropy are still in their infancy but show great potential. Importantly, ALS genetic research is accelerating, and there is hope for the future that we can create a more complete picture of disease as well as develop successful therapeutic interventions.

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Disclosure

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