Transactions on Materials, Biotechnology and Life Sciences
ISSN: 2960-1932, eISSN: 2960-2319 | Volume 7
MISBP 2024

The Application of RNA Sequencing in Understanding the Molecular Mechanisms and Therapeutic Potential in Amyotrophic Lateral Sclerosis

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Abstract. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the progressive loss of motor neurons, leading to muscle weakness and paralysis. While significant advances have been made in understanding the pathophysiology of ALS, there is still no curative treatment, and the underlying molecular mechanisms remain incompletely understood. RNA sequencing (RNA-seq) has emerged as a powerful tool to analyze the transcriptome, providing critical insights into gene expression changes, alternative splicing events, and RNA metabolism. This review highlights the application of RNA-seq in ALS research, including its use in identifying ALS-related gene mutations and differential gene expressions. Key findings include the role of C9orf72, SOD1, FUS, TARDBP, and UBQLN2 mutations in disease progression, as well as the identification of potential biomarkers in cerebrospinal fluid and peripheral blood samples. Additionally, novel RNA-seq technologies, such as single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics, are explored for their ability to capture the heterogeneity of cellular populations and unravel the complex molecular mechanisms underlying ALS. Despite the promise of RNA-seq, several technical, analytical, and ethical challenges must be addressed to fully realize its potential in ALS research. Future directions include integrating RNA-seq data with other multi-omics approaches, advancing personalized medicine, and applying emerging sequencing technologies. Overall, RNA-seq continues to play a pivotal role in uncovering the molecular mechanisms of ALS and holds promise for the development of targeted therapies.

Keywords: Amyotrophic lateral sclerosis; RNA sequencing; Gene expression; Personalized medicine.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by the death of upper and lower motor neurons, leading to muscle weakness and paralysis [1]. The pathology involves complex mechanisms, including autoimmune processes and microglial activation. ALS is categorized into familial (5-10%) and sporadic (90-95%) forms, with an estimated 16,000-20,000 cases in the United States. Diagnosis is challenging, typically taking 14 months from symptom onset, and relies on clinical signs and exclusion of ALS-like conditions [2].

ALS is unique among neurodegenerative diseases due to its selective impact on motor neurons, sparing cognitive functions. Recent research has revealed that ALS is not solely a motor disorder but also involves a range of non-motor symptoms, which could serve as potential biomarkers for disease progression [3]. However, the pathophysiology of ALS is complex and multifactorial, involving mechanisms such as excitotoxicity, neuroinflammation, mitochondrial dysfunction, and oxidative stress and that the exact cause of ALS is still unknown. Moreover, despite advances in understanding the disease mechanisms, there is still no curative treatment for ALS. Current research focuses on identifying new therapeutic targets and reliable biomarkers to aid in the development of effective treatments and improve patient management [4].

RNA sequencing (RNA-seq) is a powerful next-generation sequencing technique used to analyze the transcriptome, providing insights into gene expression, alternative splicing, and post-transcriptional modifications. This high-resolution method offers advantages over traditional microarrays, including

better quantification of lowly and highly expressed genes [5]. RNA-seq can be applied to various RNA populations, such as mRNA, miRNA, and tRNA, and can identify exon/intron boundaries. The basic workflow involves RNA isolation, cDNA synthesis, adapter attachment, library preparation, and sequencing. Recent advancements include single-cell sequencing and in situ sequencing of fixed tissue [5]. In cancer research, RNA-seq provides valuable insights into the genetic basis and progression of the disease [6].

RNA-seq analysis has revealed significant insights into neurodegenerative diseases, particularly Alzheimer's disease (AD), Parkinson's disease (PD), and ALS. These studies have identified differentially expressed genes and long non-coding RNAs (lncRNAs) in patient samples, highlighting the importance of RNA metabolism in disease pathogenesis [7]. The role of RNA-binding proteins and RNA-mediated toxicity has been emphasized in ALS, with potential implications for therapeutic approaches. Alternative polyadenylation (APA) has been found to affect genes associated with protein turnover and mitochondrial function in these diseases, suggesting a potential role in neurodegeneration [8]. Furthermore, non-coding RNAs, including circular RNAs (circRNAs), microRNAs (miRNAs), and lncRNAs, have emerged as potential biomarkers for ALS diagnosis and prognosis [9]. These findings underscore the value of RNA-seq in elucidating molecular mechanisms and identifying potential therapeutic targets in neurodegenerative diseases.

This research aims to summarize the application of RNA sequencing (RNA-seq) technology in ALS studies, analyze its significant contributions, and explore future development directions. RNA-seq, with its ability to capture the entire transcriptome, has provided critical insights into the complex molecular mechanisms of ALS, including gene expression patterns and alternative splicing events, which are crucial for understanding the disease and developing targeted therapies. By synthesizing current findings and identifying research gaps, this study seeks to guide future applications of RNA-seq in ALS, ultimately advancing efforts toward effective treatments.

2. Molecular mechanisms and gene expression in ALS

2.1. ALS-related gene mutations

The molecular mechanisms that drive neurodegeneration in ALS are intricate and involve a variety of genetic mutations and changes in gene expression. Some of the most commonly implicated genes include C9orf72, SOD1 (Superoxide Dismutase 1), FUS (Fused in Sarcoma), TARDBP (TDP-43), and UBQLN2 (Ubiquilin 2).

The C9orf72 gene mutation, which involves hexanucleotide repeat expansions, is the most prevalent genetic cause of ALS [10]. This mutation drives neurodegeneration through multiple mechanisms, including the loss of C9ORF72 protein function and a toxic gain of function due to repeat-containing RNAs and dipeptide repeat proteins. Reduced C9ORF72 levels exacerbate repeat-mediated toxicity and hinder autophagy, a critical cellular clearance mechanism [10]. Since C9ORF72 is involved in regulating the autophagy-lysosome pathway, its dysfunction, alongside other toxic mechanisms, contributes to ALS progression.

Mutations in SOD1, encoding the antioxidant enzyme superoxide dismutase, are another major contributor to ALS pathogenesis. These mutations lead to altered enzyme functionality and protein aggregation, which are associated with motor neuron death. While SOD1 mutations are typically linked to familial ALS, sporadic cases have also been reported [11]. The effects of SOD1 mutations are not limited to neurons; they also disrupt glial and skeletal muscle cells, leading to imbalances in redox states and calcium homeostasis [12].

FUS mutations cause protein mislocalization and aggregation in motor neurons, disrupting splicing factor networks and autoregulation [13]. Dysregulation of G-quadruplex-dependent liquid-liquid phase separation in FUS-mutated ALS patients leads to protein aggregation, a hallmark of the disease.

Mutations in TARDBP, encoding TDP-43, account for 2-5% of familial ALS cases [14]. TDP-43 is crucial for various cellular mechanisms, and its dysregulation leads to hyperphosphorylated and ubiquitinated deposits in motor neurons, which contribute to ALS pathology. Studies in animal models have confirmed that even physiological levels of mutant TDP-43 can induce neurotoxicity in a dose-dependent manner [15].

Finally, mutations in UBQLN2 impair protein degradation via the ubiquitin-proteasome system and autophagy, leading to motor neuron inclusions [16]. ALS-linked UBQLN2 mutations also disrupt the formation of stress-induced biomolecular condensates, exacerbating protein aggregation.

The discovery of these mutated genes provides valuable insights into the molecular mechanisms driving ALS and offers potential avenues for therapeutic intervention.

2.2. ALS-related differential gene expressions

Recent studies on differentially expressed genes (DEGs) in ALS patients have revealed complex transcriptional landscapes.

Researcher Swindell WR conducted a meta-analysis regarding DEGs. The study identified a total of 222 ALS-increased differentially expressed genes (DEGs) and 278 ALS-decreased DEGs. Among these ALS-increased DEGs, the researcher found that these genes were significantly associated with processes related to immune response, blood vessel development, and extracellular matrix (ECM) components. Specifically, they were enriched in genes linked to the collagen-containing ECM, indicating a potential role in the structural changes observed in ALS motor neurons. Moreover, these ALS-increased DEGs were also linked to pathways involving PI3K-AKT signaling, innate immunity, and inflammation, suggesting that immune processes may play a critical role in ALS pathology. Gene set enrichment analysis confirmed that these genes were enriched in terms related to the regulation of immune system processes and cellular components such as plasma membrane and exosomes. On the other hand, the ALS-decreased DEGs were primarily associated with neurogenesis, axon growth, and the ubiquitin-proteasome system, indicating a loss of essential functions in motor neurons affected by ALS. Notably, genes such as MAP kinase activating death domain (MADD) and ubiquitin specific peptidase 13 (USP13) showed significant decreases in expression across multiple datasets, highlighting their potential importance in ALS. Also, it was found that a strong majority of ALS-decreased DEGs were more highly expressed in neuronal cell types compared to other cell types, although their overall expression was lower than that of non-DEGs in other cell types. Overall, the findings suggest that ALS is characterized by both an increase in genes associated with immune and structural processes and a decrease in genes critical for neuronal function and development. This duality may contribute to the complex pathology of ALS [17].

The study conducted by Gascon *et al.* aims to conduct a comprehensive analysis of the skeletal muscle transcriptome in sporadic Amyotrophic Lateral Sclerosis (sALS) to identify differentially expressed genes (DEGs) and understand their roles in the disease. The study identified a set of differentially expressed genes in the skeletal muscle of sALS patients compared to healthy controls. Notable up-regulated hub genes included EEF1A1, RPLP0, EEF2, EIF4A1, CCT2, HNRNPR, RPL12, RPL15, HNRNPA1, and PABPC1, which are associated with protein synthesis and cellular stress responses. This finding was consistent with the previous study. Moreover, this analysis revealed significant pathways related to muscle structure development for up-regulated genes and energy metabolism for down-regulated genes. This aligns with the observed skeletal muscle dysfunction contributing to progressive muscle weakness in ALS patient [18].

In the study conducted by Dash *et al.,* researchers specifically investigated the downstream changes of motor neurons gene expressions impacted by mutations in SOD1 and TARDBP. They found out that for SOD1-mutatedALS patients, there was a significant downregulation of genes involved in protein translation and mitochondrial function, suggesting a shift in cellular RNA abundance profiles that could contribute to neural dysfunction. Also, for TARDBP-mutated ALS patients, the transcripts encoding components of the transcriptional machinery and those involved in splicing regulation were

particularly affected, indicating that RNA processing mechanisms play a crucial role in this form of ALS [19].

Overall, different studies have provided insights into the molecular changes occurring in neurons or muscle molecules affected by ALS. All of the information related to molecular changes could provide insights to ALS mechanisms and inform future therapeutic strategies.

3. Application of RNA-seq in ALS

RNA sequencing (RNA-seq) has become an essential tool for understanding the pathogenesis of ALS, allowing researchers to investigate the transcriptome at unprecedented depth. The ability of RNA-seq to capture the full spectrum of RNA transcripts has led to numerous discoveries about gene expression changes, RNA processing errors, and potential therapeutic targets in ALS research.

3.1. Application of RNA-seq in ALS patient sample

RNA-seq offers a diverse range of applications in ALS research, including gene expression profiling, identifying pathogenic mutations, detecting alternative splicing events, discovering non-coding RNAs, and searching for reliable biomarkers. By providing a comprehensive view of the transcriptome, RNA-seq has facilitated the identification of novel disease mechanisms.

For instance, RNA-seq has been extensively utilized to profile gene expression in peripheral blood mononuclear cells and patient-derived motor neurons, revealing significant differences in gene expression between ALS patients and healthy controls [20]. These studies have identified dysregulation in key pathways, including transcriptional machinery, RNA splicing regulation, and mitochondrial dysfunction, all of which play crucial roles in ALS progression. Furthermore, RNA-seq studies using patient-derived spinal cord samples have allowed researchers to track transcriptomic changes over time, identifying key steps in motor neuron degeneration. Co-expression network analyses have further highlighted the role of RNA processing, intracellular transport, and autophagy, implicating various cell types, such as astrocytes, oligodendrocytes, and microglia, in the disease's progression [21].

RNA-seq has also proven useful for detecting splicing abnormalities. For example, Nakaya [22] identified a gene-splicing alteration in the SNRNP70 gene as a hallmark of a specific ALS subtype. Likewise, Wang *et al.* developed the SpliPath computational framework to detect intronic mutation hotspots in the KIF5A gene, which could contribute to ALS pathogenesis. These findings demonstrate the utility of RNA-seq in uncovering not only gene expression changes but also crucial splicing errors, which could open up new avenues for therapeutic intervention.

In the search for ALS biomarkers, RNA-seq has been invaluable. By analyzing cerebrospinal fluid (CSF) and blood samples from ALS patients, researchers have identified potential biomarkers that could improve diagnosis and disease monitoring. Fröhlich *et al.* found differential gene expression in CSF, revealing genes such as CAPG, CCL3, and MAP2 as potential biomarkers [23]. Similarly, Joilin *et al.* identified a non-coding RNA signature in serum, including microRNAs and transfer RNAs, that could differentiate ALS patients from healthy individuals with high accuracy. These biomarkers not only improve diagnostic precision but also offer a means to track disease progression and therapeutic response.

3.2. Single-cell RNA-seq and its application in ALS

Single-cell RNA sequencing (scRNA-seq) has revolutionized ALS research by enabling the analysis of transcriptomes at the level of individual cells. This technique allows researchers to unravel the heterogeneity of cellular populations and provides a more nuanced understanding of gene expression dynamics within specific cell types.

In ALS research, scRNA-seq has been instrumental in identifying cell type-specific transcriptomic alterations. For instance, in studies using SOD1 mutant mouse models, scRNA-seq revealed critical

pathways implicated in ALS pathogenesis, including immune response and autophagy regulation [24]. Human-induced pluripotent stem cell (iPSC)-derived motor neurons have also been studied using scRNA-seq, providing insights into early transcriptomic signatures common to both familial and sporadic ALS [25]. These studies showed downregulation of genes such as ELAVL3, which plays a role in neuronal health and function. Furthermore, scRNA-seq has enabled the identification of distinct neural subtypes and developmental stages in ALS models, offering new insights into how motor neurons are specifically affected in ALS [25].

This technique has also been pivotal in revealing transcriptional drivers of neurodegeneration, such as the activation of the TGF-β signaling pathway via SMAD2. The discovery of this pathway, which has been linked to neuroinflammatory responses in ALS, highlights the potential of scRNA-seq in identifying novel therapeutic targets [26]. As scRNA-seq continues to advance, its ability to pinpoint cell-specific vulnerabilities in ALS could lead to more targeted and personalized therapeutic approaches.

4. Challenge and limitation of RNA-seq in ALS research

Despite its numerous advantages, RNA sequencing faces several challenges and limitations in the context of ALS research. These challenges span technical, analytical, and ethical domains, all of which must be addressed to maximize the utility of RNA-seq in ALS studies.

4.1. Challenge of RNA-seq in the technical aspect

One of the major technical hurdles in RNA-seq research is the difficulty in obtaining high-quality tissue samples. Since ALS primarily affects motor neurons located in the spinal cord and brain, accessing these tissues from living patients is extremely challenging. Consequently, most studies rely on post-mortem samples, which can suffer from RNA degradation and may only reflect the disease's late stages. This limitation hampers the ability to study the early molecular changes that occur in ALS, limiting our understanding of disease onset and progression.

Another challenge arises from the heterogeneity of ALS itself. ALS is a highly variable disease, with significant differences in clinical manifestations, genetic backgrounds, and underlying pathogenic mechanisms. This heterogeneity complicates the interpretation of RNA-seq data, as different ALS subtypes may show distinct gene expression patterns. As a result, RNA-seq studies may require larger sample sizes or more sophisticated stratification techniques to capture the full range of ALS variability and provide meaningful insights for all patient groups.

Finally, the complexity of RNA-seq data analysis presents another obstacle. RNA-seq generates vast amounts of data that require extensive computational resources and bioinformatics expertise for proper interpretation. Even small variations in data processing pipelines, such as differences in RNA extraction methods, sequencing platforms, or algorithmic approaches, can lead to inconsistent results. For instance, Grima *et al.* found that RNA-seq models for predicting disease duration in ALS patients performed poorly, with a significant mean prediction error of nearly two years [27]. This highlights the need for standardized protocols and robust validation across independent datasets to improve RNA-seq's predictive accuracy.

4.2. Ethical Considerations

Ethical issues in ALS research, particularly regarding sample acquisition, are another significant barrier to progress. Given the rarity and rapid progression of ALS, obtaining sufficient patient samples—especially from early-stage patients—remains a significant challenge. Invasive procedures, such as biopsies of the brain or spinal cord, are often required to obtain high-quality samples, but these methods are ethically contentious and not easily accepted by patients or their families.

Moreover, as RNA-seq technology can reveal comprehensive gene expression profiles, concerns about patient privacy and data security are paramount. The potential for privacy breaches, particularly

when sharing transcriptomic data through public repositories, necessitates stringent ethical guidelines. Researchers must ensure that patient consent is fully informed, particularly regarding the long-term use of their genetic data.

Additionally, the legal landscape governing the use of human samples varies across countries, complicating international collaboration in ALS research. Differing regulations on sample acquisition and data sharing can slow the pace of research and limit the scope of global studies aimed at finding new ALS therapies and biomarkers.

5. Future Research Directions of RNA-seq Application in ALS Research

5.1. Integrative Analysis Combining Multi-Omics Data

The integration of multi-omics approaches—combining genomics, transcriptomics, proteomics, and metabolomics—has gained momentum in improving our understanding of complex diseases, including ALS. RNA-seq, in this context, can be combined with other omics techniques such as ATAC-seq (to map chromatin accessibility) and ChIP-seq (to analyze protein-DNA interactions) to offer a multi-dimensional view of gene regulation and molecular changes associated with ALS. Recent computational tools have facilitated the integration of these data types, offering a more comprehensive analysis of cellular processes.

The integration of RNA-seq with other omics layers brings several key advantages. First, it provides a more complete molecular landscape, allowing for a thorough understanding of gene expression, regulatory networks, and functional protein changes. While RNA-seq alone can identify gene expression changes, integrating it with genomics and epigenomics can reveal the upstream factors controlling these changes, such as mutations or epigenetic modifications like DNA methylation and histone alterations. For instance, when integrated with ATAC-seq data, RNA-seq can help researchers understand how chromatin accessibility affects gene expression, linking transcriptomic alterations to regulatory elements.

Second, multi-omics approaches enable cross-validation of findings. RNA-seq data, for example, can be cross-referenced with proteomics to determine whether changes in mRNA levels correspond to changes in protein abundance, thereby confirming the functional consequences of observed gene expression changes. A study by Wang *et al.* demonstrated how integrating single-cell RNA-seq and ATAC-seq data improved accuracy in characterizing molecular changes by 55.7%, highlighting the potential of multi-omics approaches to provide more accurate disease models [28].

Finally, combining RNA-seq with proteomics or metabolomics enhances our understanding of disease mechanisms and facilitates drug discovery. Multi-omics approaches can reveal how genetic and transcriptomic changes translate into altered protein function and metabolic pathways, providing a holistic view of the effects of therapeutic interventions. This comprehensive analysis is particularly valuable in drug development, offering insights into both therapeutic efficacy and potential side effects.

5.2. Application of Novel RNA Sequencing Technologies

In recent years, novel RNA-seq technologies, such as single-cell RNA-seq (scRNA-seq), spatial transcriptomics, single-nucleus RNA-seq (snRNA-seq), and ribo-depleted RNA-seq, have emerged as powerful tools in transcriptomic research. These advanced techniques allow for more precise and comprehensive profiling of gene expression, offering significant potential in ALS research.

Spatial transcriptomics is an innovative technique that combines RNA-seq with spatial information, enabling researchers to study gene expression in the context of tissue architecture. While this technology has proven highly valuable in understanding neurological diseases such as Alzheimer's disease (AD), its use in ALS research remains limited. However, it holds immense potential. For example, spatial transcriptomics has been used to identify ALS-associated genes such as *NOMO1*,

which is enriched in specific regions of the motor cortex, offering new insights into the spatial distribution of gene expression in ALS [29]. Future ALS studies should aim to apply spatial transcriptomics to further investigate the cellular and molecular landscapes of affected tissues.

Single-nucleus RNA-seq (snRNA-seq) is another promising technique that allows transcriptomic profiling of frozen or difficult-to-dissociate tissues, making it particularly suitable for studying ALS-affected motor neurons in the brain and spinal cord. This method has enabled the study of post-mortem ALS patient samples, revealing molecular disruptions specific to different cell types, such as neurons and glial cells. Given the challenges of obtaining live tissue from ALS patients, snRNA-seq presents a valuable tool for studying archived samples, offering new insights into the transcriptomic changes associated with ALS progression.

Ribo-depleted RNA-seq, also known as total RNA-seq, provides a comprehensive view of both coding and noncoding RNA molecules by removing ribosomal RNA (rRNA) from samples. This technique allows for the detection of splicing abnormalities and the analysis of noncoding RNAs, such as long noncoding RNAs (lncRNAs), which play important roles in ALS pathogenesis. Despite its potential, ribo-depleted RNA-seq has not been widely applied in ALS research. Given its ability to capture a broader range of RNA species, future studies should explore this technique to gain a deeper understanding of RNA metabolism dysfunction in ALS.

5.3. Personalized medicine

Personalized medicine, an approach that tailors' treatments based on individual patients' genetic and molecular profiles, has the potential to revolutionize ALS treatment. As a heterogeneous disease with multiple genetic mutations and variations, ALS presents an ideal candidate for personalized therapeutic strategies. RNA-seq plays a critical role in this approach by enabling the identification of molecular subtypes and individual gene expression profiles, which can guide treatment decisions.

RNA-seq allows researchers to stratify ALS patients based on their unique molecular signatures, enabling more precise therapeutic interventions. For example, RNA-seq has identified specific molecular pathways altered in ALS subtypes, such as the RNA processing defects seen in patients with TARDBP or FUS mutations [30]. These insights have already paved the way for targeted therapies, such as antisense oligonucleotides (ASOs) designed to suppress mutant forms of *SOD1* or *C9orf72*. Moving forward, integrating RNA-seq with clinical practice could help clinicians select the most effective treatments based on an individual patient's gene expression profile, facilitating the shift from reactive to preventative and targeted care.

To realize the full potential of personalized medicine, future studies should focus on translating RNA-seq findings into clinical applications. While current research has identified potential biomarkers and therapeutic targets, their clinical implementation remains limited [30]. The integration of RNA-seq with emerging therapeutic approaches, such as gene editing and immunotherapies, holds the promise of delivering personalized, curative treatments for ALS patients.

6. Conclusion

RNA sequencing (RNA-seq) has profoundly advanced our understanding of Amyotrophic Lateral Sclerosis (ALS), shedding light on the molecular mechanisms underlying this devastating neurodegenerative disease. By providing comprehensive insights into gene expression, RNA-seq has revealed the complex transcriptional landscape of ALS, identifying key gene mutations and differential gene expressions that contribute to disease pathogenesis. The technology has also been instrumental in uncovering splicing abnormalities and noncoding RNA functions, offering new avenues for therapeutic development.

Despite significant progress, several challenges remain. The integration of RNA-seq with other omics data, such as genomics and proteomics, will be crucial for gaining a holistic view of ALS pathology and developing targeted therapies. Future research should also focus on exploring novel RNA-seq

technologies, such as spatial transcriptomics and single-nucleus RNA-seq, to further refine our understanding of the disease at the cellular level. Moreover, advancing the field of personalized medicine through the application of RNA-seq findings could pave the way for more effective and individualized treatment strategies.

In conclusion, RNA-seq continues to be a powerful tool in ALS research, with immense potential to drive future discoveries and therapeutic breakthroughs. As researchers further refine these technologies and integrate them with other cutting-edge approaches, we can expect significant strides toward unraveling the complexities of ALS and developing more effective treatments.

Acknowledgements

I would like to express my sincere gratitude to Li for his invaluable guidance, insightful feedback, and continuous support throughout this research. I am also grateful to my colleagues and friends for their encouragement and assistance. Lastly, I would like to thank my family for their unwavering support and patience during this process.

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