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Research Article

Identifying miRNAs associated with amyotrophic lateral sclerosis and mitochondrial dysfunction through bioinformatics approaches

Gulcin Baykal, Burcin Erkal, Senay Vural Korkut

Department of Molecular Biology and Genetics, Yildiz Technical University Faculty of Arts and Sciences, Istanbul, Turkiye

Abstract

Objectives: MicroRNAs (miRNAs) are non-coding RNA molecules that control gene expression by causing messenger RNA to degrade after transcription. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that involves the gradual deterioration and death of motor neurons, resulting in muscle weakness, paralysis, and eventual fatality. A prominent feature of ALS is the presence of mitochondrial dysfunction. The identification of miRNAs associated with mitochondrial dysfunction in ALS through *in silico* approaches can offer valuable insights into underlying processes and targets for therapeutic interventions.

Methods: Computational tools and publicly available databases were employed for the purpose of identifying specific miRNAs that could potentially play a role in the onset or progression of mitochondrial dysfunction in ALS.

Results: A review of the literature revealed that the genes *SOD1*, coiled-coil-helix-coiled-coil-helix domain-containing protein 10, *C9orf72*, *OPTN*, valosin-containing protein, *TARDBP, TBK1, FUS*, and *BCL2* are linked to mitochondrial dysfunction and are involved in the etiology of ALS. Enrichment analyses of biological pathways performed with the computational tools Enrichr, g: Profiler, and CROssBAR confirmed that the discovered genes are strongly related to ALS, mitochondrial failure, and differentiation of neurons. Furthermore, miRNA computational predictions were performed utilizing the publicly available databases miRTargetLink, miRNet, miRWalk, and TargetScan. To identify common miRNAs, a Venn diagram was used, and 28 miRNAs were selected as a result. For further analysis, a set of 28 miRNAs was subjected to functional and enrichment studies using the computational programs miRNet and TAM, respectively. Results from both computational programs consistently revealed that the miRNAs hsa-miR-125b, hsa-miR-9-5p, and hsa-miR-141-3p were related to ALS.

Conclusion: We proved that *in silico* approaches projected the effects of miR-9, miR-141, and miR-125b targeting the identified genes on ALS, and we demonstrated that the genes involved in mitochondrial dysfunction in ALS are based on the literature.

Keywords: Amyotrophic lateral sclerosis, biomarkers, miRNAs, mitochondrial dysfunction

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Amyotrophic lateral sclerosis (ALS) is a lethal neurodegenerative disorder marked by the degeneration of both upper and lower motor neurons. This condition results in progressive muscular atrophy that impacts motor neurons situated in various regions of the central nervous system, including the spinal cord, brainstem, and cerebral cortex. As a result, the afflicted individual may experience muscle twitches and the disconnection of neurons from the target muscles they control. ALS typically develops in people in their late middle age [1, 2]. The majority of ALS cases, approximately 90%, are of the sporadic form. Sporadic ALS (sALS) develops without any identifiable family history or genetic inheritance.

Address for correspondence: Senay Vural Korkut, PhD. Department of Molecular Biology and Genetics, Yildiz Technical University Faculty of Arts and Sciences, Istanbul, Türkiye
Phone: +90 533 414 39 93 **E-mail:** skorkut@yildiz.edu.tr **ORCID:** 0000-0002-6260-0357
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In contrast, familial ALS (fALS) is a less common form of disease that results from genetic mutations in specific genes. Approximately 5–10% of all cases are this type. The incidence of the disease varies from 0.6 to 3.8 cases/100,000 individuals, while the prevalence ranges from 4.1 to 8.4 cases/100,000 individuals [3]. It has been observed that the incidence and prevalence of the disease rise in lockstep with age [2].

ALS pathophysiology involves various cellular and molecular processes, such as abnormalities in RNA metabolism, accumulation of toxic protein aggregates, impaired mitochondrial function, excitotoxicity, disruption of axonal transport, oxidative stress, and neuroinflammation. Motor neuron degeneration is attributed to these processes, each of which can contribute to the breakdown of motor neurons, resulting in the loss of muscle control and muscle wasting [4].

Mitochondrial dysfunction has been associated with the onset of some diseases such as diabetes, cardiovascular disease, and cancer [5, 6]. Mitochondrial dysfunction impacts not just the energy production process of mitochondria, but also other cellular processes such as mitophagy, calcium signaling, and protein processing in the endoplasmic reticulum (ER) [7, 8]. Due to their significant energy requirements and the need for ATP synthesis and Ca2+ regulation during synaptic transmission, neurons predominantly depend on oxidative metabolism, leading to considerable demand for a large quantity of mitochondria. Furthermore, these cells are characterized by high polarity, featuring long axons that necessitate active transport of mitochondria to maintain their functionality, which contributes to their high mitochondrial content. However, the process of oxidative metabolism produces reactive oxygen species (ROS), which can damage mitochondrial DNA and proteins, resulting in a decline in mitochondrial function [7, 8].

Extensive research consistently links mitochondrial dysfunction to the development of ALS. Studies in animal models and human samples reveal various impairments, including defects in mitochondrial dynamics, respiratory chain function, disrupted calcium homeostasis, and alterations in mitochondrial DNA. For example, Wiedemann et al. [9] found abnormal mtDNA content and oxidative phosphorylation complexes in motor neurons of ALS patients, supporting the link between mitochondrial dysfunction and ALS neurodegeneration [9]. The research conducted by the Magrane group is also crucial in understanding this connection. They demonstrated that mutant *SOD1* disrupts mitochondrial dynamics, leading to bioenergetic dysfunction and synaptic alterations in ALS motor neurons [10]. Another study by Magrane et al. [11] revealed abnormal mitochondrial transport and morphology in ALS mouse models expressing mutant *SOD1* or *TDP43*, suggesting shared mitochondrial dysfunction as a pathological feature in ALS.

Research has demonstrated that the aggregation of mutant *SOD1* within neuronal mitochondria affects mitochondrial morphology. In addition, mutant *SOD1* proteins aggregate together in mitochondria with Bcl-2, an antiapoptotic protein. This interaction may contribute to mitochondrial dysfunction and neuronal death in ALS [12].

Mutant *TDP-43* proteins have been observed to form aggregates in both the cytosol and mitochondria of neurons, as well as to damage the ETC subunit complex I. Furthermore, the presence of mutant *TDP-43* may make these aggregates more susceptible to oxidative stress [13]. Mutations in the *FUS* gene have been linked to neurodegeneration and functional changes at the neuromuscular junction (NMJ), resulting in enlarged mitochondria with misaligned cristae and membrane structures. Muscle degeneration can also occur because of mitochondrial dysfunction in the NMJ, resulting in an inability to stimulate proper muscle function [14]. Research has indicated that mutant *C9orf72*, which is linked to ALS, can bind to mitochondrial proteins and lead to mitochondrial dysfunction. This can result in an increase in oxidative stress, an effect on mitochondrial membrane potential, reduced *Bcl-2* levels, and alterations in mitochondrial morphology [15]. Mutant Coiled-coil-helix-coiled-coil-helix domain-containing protein 10 (*CHCHD10*) induces mitochondrial abnormalities, such as fragmentation and misregulated cristae structure, contributing to ALS. The ensuing stress from these malfunctions leads to motor neuron death [16]. Mutant Valosin-containing protein (*VCP*) associated with ALS affects PINK1/Parkin-mediated mitophagy, resulting in impaired mitophagy, reduced mitochondrial oxygen consumption, decreased cellular energy capacity, and increased vulnerability to cellular processes [17, 18]. *Bcl2*, a protein known as B-cell lymphoma/leukemia 2, is involved in the regulation of several cellular processes, including autophagy, apoptosis, and mitochondrial dynamics [19]. According to research, *Bcl2* depletion in deep motor neurons can result in severe motor neuron losses, which is critical for conserving certain neuronal populations throughout the early postnatal period [20].

The critical biochemical significance of microRNAs (miRNAs) stems from their pivotal role in regulating gene expression at the post-transcriptional level in eukaryotic cells. miRNAs are single-stranded, non-coding RNA molecules that could bind to messenger RNAs (mRNAs), thereby impeding their translation into proteins and silencing genes. This process is critical for maintaining cellular homeostasis and controlling gene expression. They can target and regulate multiple mRNA molecules, while a single mRNA can be controlled by several miRNAs [21, 22]. miRNAs play a vital role in multiple cellular processes that are essential for the proper functioning of the nervous system, such as neuronal differentiation, neurogenesis, and synaptic plasticity [23]. Aberrant expression of miRNAs can disrupt these processes, leading to the onset and progression of neurological disorders.

The process of diagnosing ALS can be time-consuming, taking approximately 12 months on average [2]. As a result, research into ALS biomarkers is critical to aid in early diagnosis and treatment initiation [24]. The intricacy of gene expression

in ALS-related pathways has made it difficult to establish validated conventional biomarkers for diagnosing or monitoring ALS disease progression. In this study, we utilized *in silico* analyses to identify miRNAs that are specifically linked to mitochondrial dysfunction in ALS. These miRNAs were identified based on their target genes, which are crucial for both mitochondrial dysfunction and ALS. The objective of this study is to provide evidence that the identification of common miRNAs through bioinformatics can serve as promising biomarkers for streamlining and accelerating the diagnosis of ALS.

Materials and Methods

Literature search and data extraction

The literature search was conducted by utilizing the Science Direct and PubMed databases, with a focus on studies published up until 2022. To identify eligible studies, the researchers used specific MeSH terms related to the topic, including miRNAs, mitochondria, biomarkers, and ALS.

Pathway enrichment analyses

The identified genes from the literature search were subjected to gene set enrichment analysis using the Enrichr tool available at https://maayanlab.cloud/Enrichr/ (last accessed, May 2023). The Kyoto Encyclopedia of Genes and Genomes (KEGG) 2021 Human pathway analysis was employed to analyze the identified genes. The g: Profiler tool, accessible at https://biit.cs.ut.ee/gprofiler/gost (last accessed, May 2023), was also used to analyze the genes, where biological pathway analyses were performed using KEGG and WikiPathway in g: Gost functional profiling. Finally, the CROssBAR tool, available at https://crossbar.kansil.org (last accessed, May 2023), was used to analyze the genes.

Prediction of miRNAs

Four different databases were used to obtain more specific results for identifying miRNAs that target the determined genes These are miRWalk (http://mirwalk.umm.uni-heidelberg.de, last accessed, May 2023), miRTargetLink 2.0 (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2, last accessed, May 2023), miRNet 2.0 (https://www.mirnet.ca, last accessed, May 2023), and TargetScan (https://www.targetscan.org/vert_80/, last accessed, May 2023). To identify miRNAs related to specific genes, a comprehensive search was conducted for Homo sapiens in multiple databases. Each identified gene was subsequently searched for separately in four different databases. To visualize miRNAs commonly associated with each gene, a Venn diagram tool was used (https://bioinformatics.psb.ugent.be/webtools/Venn/, last accessed, May 2023). The inclusion criteria for this study required miRNAs to be identified in at least two of the four databases for each gene. Common miRNAs that targeted at least three genes were compiled into a single list (Appendix 1). For all analyses, a $p<0.05$ was considered statistically significant.

Set analyses of miRNAs

Following the compilation of the common miRNA list, further analysis was conducted using two online tools: miRNet 2.0 (https://www.mirnet.ca, last accessed, May 2023) and TAM 2.0 (http://www.lirmed.com/tam2/, last accessed, May 2023). These tools were utilized to perform set analysis on the miRNAs, with the aim of identifying those that are associated with specific tissue types, functions, and disease areas, based on previous enrichment analyses. A Venn diagram was employed to visualize the common miRNAs that were identified by both databases.

Results

Gene selection

Figure 1 provides an overview of the study. A meticulous examination of the scientific literature was conducted to unveil potential genes associated with mitochondrial dysfunction in ALS (Fig. 1). Through this review, a total of nine genes emerged as potential candidates. These genes are *SOD1*, *CHCHD10, C9orf72, OPTN, VCP, TARDBP, TBK1, FUS*, and *BCL2*. A subsequent investigation of these genes was carried out, and the ensuing outcomes, along with references, are delineated in Table 1. Utilizing a KEGG Pathways map, we visually represented the plausible engagement of these genes in ALS-related pathways (Fig. 2). The pertinent genes within the map were highlighted in yellow, underscoring their involvement in diverse biological processes associated with the disease.

Pathway enrichment analyses of determined genes

Upon analysis using the Enrichr database, it was determined that the chosen genes exhibited a significant association with ALS, as indicated in Table 2. Seven of these genes were also found to be significantly linked to neurodegeneration. *TBK1* and *OPTN* were discovered to be highly connected with mitophagy, whereas *TBK1* and *BCL2* were discovered to be associated with autophagy (Table 2). The KEGG pathway analysis on nine genes was performed using the g: Profiler tool, and the results revealed that these genes were substantially related to ALS and eight of these genes were significantly linked to neurodegeneration (Fig. 3). The study also revealed multiple genes involved that mediate abnormal mitochondrial morphology, autophagy, neuron atrophy, and morphological abnormalities, as illustrated in Figure 3. Pathway enrichment analysis with the CROssBAR tool was used to examine the genes, and the results revealed that the *TBK1* and *OPTN* genes were involved in the mitophagy pathway, and the *TBK1, BCL2*, and *C9orf72* genes were involved in the autophagy pathway (Fig. 4). The *BCL2* gene was also related to the p53 signaling pathway and apoptosis. The analysis of the *BCL2* and *VCP* genes in concert has unveiled their shared involvement in the process of protein processing within the ER, as depicted in Figure 4. In addition, the KEGG pathway analysis illustrating the intricate connections between the p53 signaling pathway, protein processing within the ER, and their interactions with ALS is delineated in Figure 2.

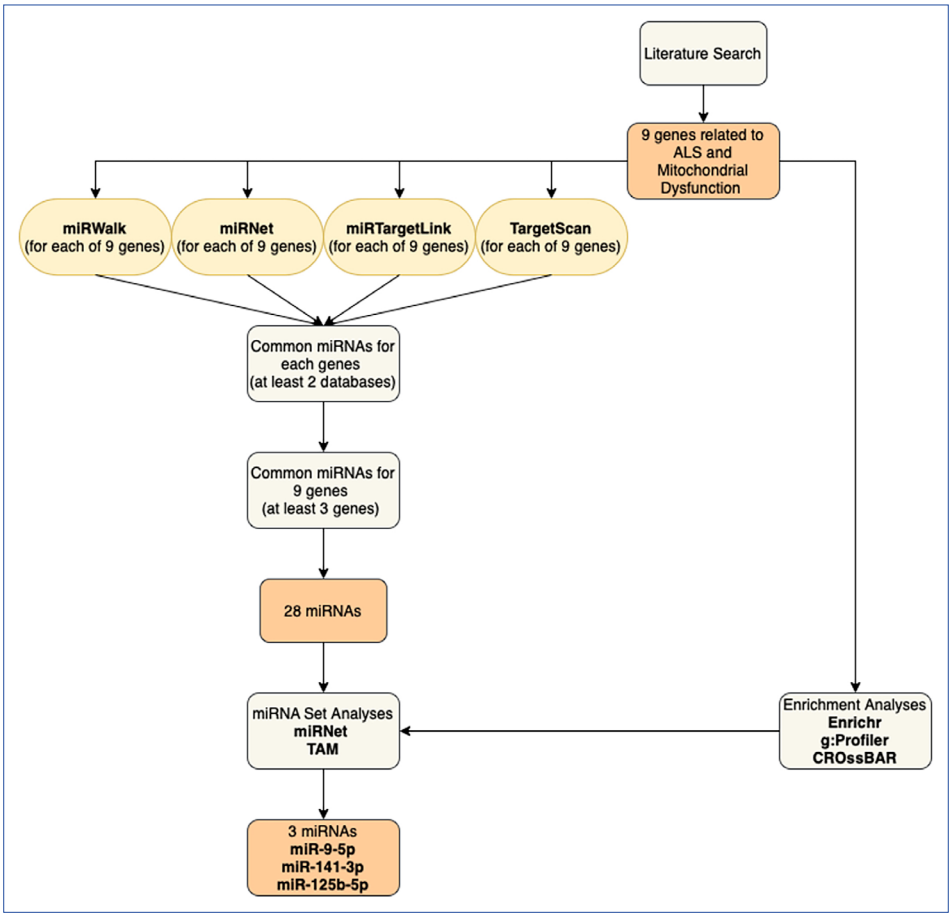

Figure 1. Overview of study.

ALS: Amyotrophic lateral sclerosis; TAM: Tool for miRNA set analysis (http://www.lirmed.com/tam2/).

Identification of miRNAs and set analyses

Rather than relying on a single database, four distinct databases were initially used to generate more results. For each gene, data were acquired independently from all four databases, and a Venn diagram was used to identify miRNAs that were present in at least two databases (Fig. 5). According to the findings, among the common miRNAs detected in at least two databases for each gene, 28 miRNAs targeting at least three genes were identified, and these miRNAs were listed using a Venn diagram (Fig. 5 and Appendix 1).

The previously identified 28 miRNAs were subsequently examined utilizing the miRNet 2.0 and TAM 2.0 tools for miRNA set analysis (Table 3). Certain miRNAs were eliminated using pathway enrichment analysis. The pathways linked with the nine discovered genes were compared to the pathways related to the 28 miRNAs. Three miRNAs were found to be important in the

Table 1. Genes are associated with mitochondrial dysfunction in ALS

Locus	Gene (Protein)	Refs
ALS1	*SOD1* (Superoxide dismutase 1)	Kong & Xu, 1998; Vande Velde et al., 2011
ALS6	*FUS* (RNA-binding protein *FUS*)	Sharma et al., 2016
ALS10	*TARDBP* (TAR DNA-binding protein)	W. Wang et al., 2016; Zuo et al., 2021
ALS12	*OPTN* (Optineurin)	Wong & Holzbaur, 2014; Liu et al., 2018
FTD/ALS6	*VCP* (valosin-containing protein)	Johnson et al., 2010; N. C. Kim et al., 2013
FTD/ALS1	*C9ORF72* (chromosome 9 Open Reading Frame 72)	Dafinca et al., 2016
FTD/ALS2	*CHCHD10* (coiled-coil-helix-coiled-coil-helix domain-containing 10)	Bannwarth et al., 2014; Genin et al., 2019
FTD/ALS4	*TBK1* (TANK Binding Kinase 1)	Harding et al., 2021
18q21.33	*BCL2* (B-cell Lymphoma 2)	Mu et al., 1996; Pedrini et al., 2010

ALS: Amyotrophic lateral sclerosis; FTD: Frontotemporal dementia.

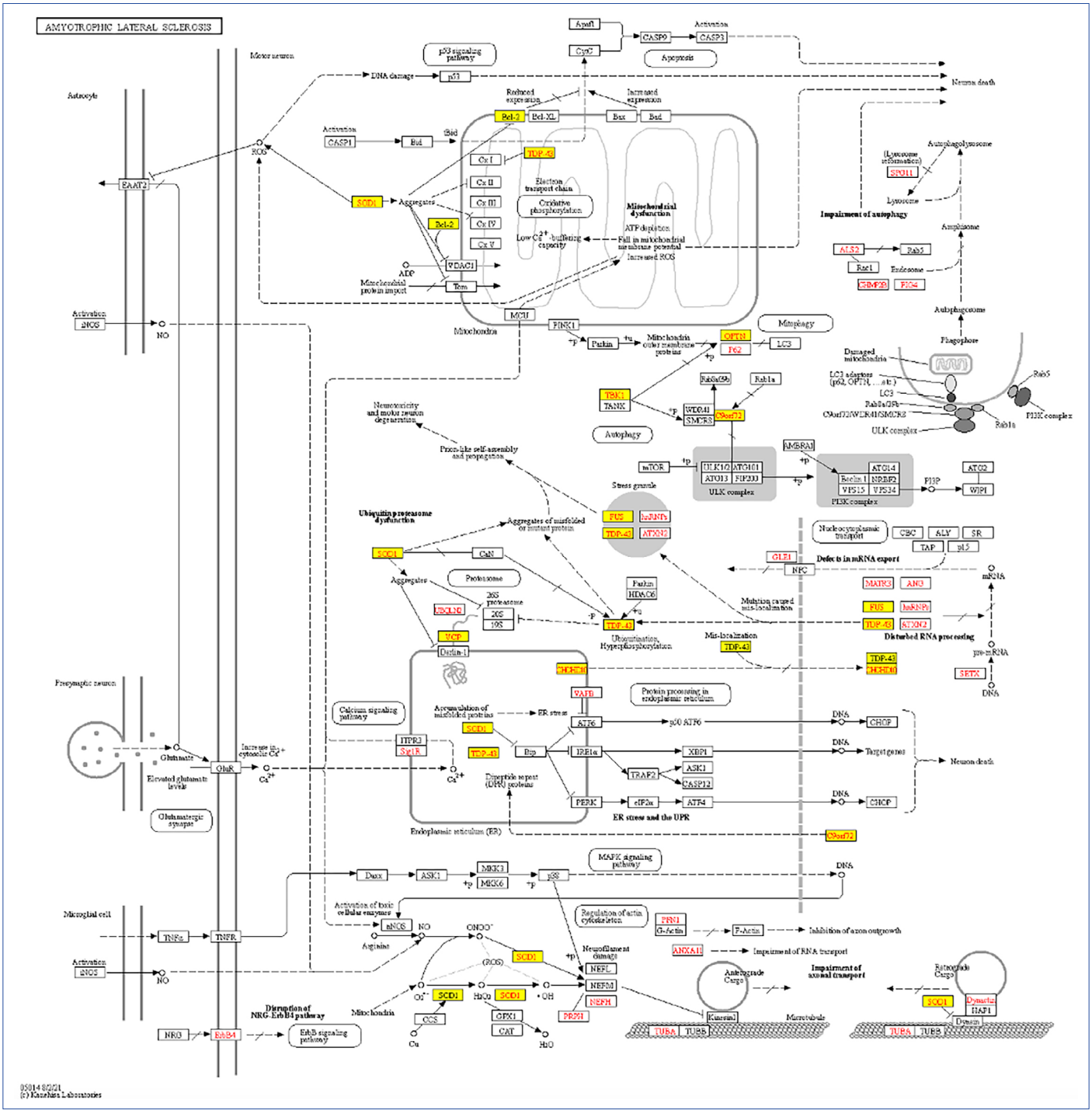

Figure 2. KEGG, Map05014, ALS pathway. The nodes highlighted in yellow indicate genes that are associated with mitochondrial dysfunction in ALS.
KEGG: Kyoto Encyclopedia of Genes and Genomes; ALS: Amyotrophic lateral sclerosis.

study: miR-9, miR-141, and miR-125. In both databases miRNet and TAM, these three miRNAs were found to be significantly associated with ALS disease, and brain tissues (Table 3 and Fig. 1).

Discussion

The identification of reliable biomarkers is critical for the accurate and timely diagnosis and effective treatment of ALS. *In silico* studies are especially beneficial for discovering possible biomarkers with increased specificity. However, there are some limitations about *in silico* methods. The purpose of this analysis was to find miRNAs that are common across all four databases, which would increase their likelihood of playing a significant role in regulating the expression of genes related to ALS and mitochondrial dysfunction. Initially, a comprehensive literature review was conducted in this study to identify nine target

Table 2. KEGG 2021 pathway enrichment analysis using enrichr tool

Term	p value	q value	Overlap genes
Amyotrophic lateral sclerosis	9.877965e-14	6.025558e-12	[*VCP, TBK1, FUS, BCL2, CHCHD10, TARDBP, OPTN, SOD1*]
Pathways of Neurodegeneration	1.409405e-10	4.298684e-09	[*VCP, TBK1, FUS, BCL2, TARDBP, OPTN, SOD1*]
Mitophagy	4.037790e-04	8.210173e-03	[*TBK1, OPTN*]
mRNA surveillance pathway	8.365989e-04	1.275813e-02	[*FUS, TARDBP*]
Autophagy	1.624891e-03	1.699943e-02	[*TBK1, BCL2*]
Measles	1.672075e-03	1.699943e-02	[*TBK1, BCL2*]
Hepatitis B	2.261359e-03	1.809705e-02	[*TBK1, BCL2*]
Protein processing in endoplasmic reticulum	2.515130e-03	1.809705e-02	[*VCP, BCL2*]
NOD-like receptor signalling pathway	2.812236e-03	1.809705e-02	[*TBK1, BCL2*]
RNA transport	2.966730e-03	1.809705e-02	[*FUS, TARDBP*]

KEGG: Kyoto Encyclopedia of Genes and Genomes; VCP: Valosin-containing protein; TBK1: TANK Binding Kinase 1; FUS: Fused in sarcoma; BCL2: B-cell Lymphoma 2; CHCHD10: Coiled-coil-helix-coiled-coil-helix domain-containing 10; TARDBP: TAR DNA-binding protein; OPTN: Optineurin; SOD1: Superoxide dismutase 1; NOD: Nucleotide oligomerization domain.

genes related to ALS and mitochondrial dysfunction. Then *in silico* methods were used to identify miRNAs that are likely to regulate the expression of these genes. For each of the nine target genes, Venn diagrams were utilized to analyze the overlap of miRNAs collected from four distinct databases. After conducting the Venn diagram analysis, a set of 28 miRNAs were identified. The identification of at least three genes that were targeted by each of the 28 miRNAs supports the findings. Three distinct tools, Enrichr, g: Profiler, and CROssBAR, were used to conduct pathway analyses to study the roles of these certain genes. Using set analysis, three miRNAs out of 28 were discovered to be statistically significant relevant to disease pathogenesis.

The pathophysiology of ALS has been studied to determine the real cause of motor neuron death and mitochondrial dysfunction has been identified as a potential contributing factor. The first evidence linking mitochondrial dysfunction to the development of ALS was reported by Bowling et al [25]. Their findings provide preliminary evidence for the potential role of mitochondrial dysfunction in ALS . Extensive research has linked mitochondrial dysfunction in ALS to *SOD1, CHCHD10, C9orf72, OPTN, VCP, TARDBP, TBK1*, *FUS*, and *BCL2* (Table 1).

Our study demonstrated a significant association between *C9orf72* and *BCL2* with autophagy using pathway enrichment analyses. Mitophagy, autophagy, and mitochondrial dysfunction are interconnected processes crucial for maintaining mitochondrial health. Autophagy is a cellular mechanism responsible for degrading and recycling cellular components [26]. Mitochondrial dysfunction refers to impaired mitochondrial function [27]. The association of ALS-related genes with mitochondrial dysfunction and autophagy highlights their relevance in neurodegenerative diseases like ALS. Autophagy's role in maintaining cellular balance and clearing damaged mitochondria makes it significant in ALS and related disorders. The association between *C9orf72* and autophagy in the context of neurodegenerative diseases, specifically ALS and FTD, has been the subject of extensive research. Webster et al. [28] identified *C9orf72*'s role in autophagy initiation through Rab1a and ULK1 complex. Sellier et al. [29] showed *C9orf72* loss impairs autophagy, leading to protein accumulation and motor neuron dysfunction. Shi et al. [30] linked toxic DPRs from *C9orf72* repeat expansion to autophagy disruption and ALS/FTD pathogenesis.

The association of *BCL2* with autophagy has also been extensively studied, and it has been found to have complex and context-dependent effects on the autophagy process. Pattingre et al. [31] showed that *BCL2* inhibits autophagy by interacting with Beclin 1, a key autophagy regulator. Wei et al. [32] demonstrated that JNK1-mediated *BCL2* phosphorylation regulates autophagy during nutrient deprivation. *Bcl-2* family proteins play a crucial role in regulating the mitochondrial metabolic pathway during apoptosis [33, 34]. Despite its roles in apoptosis, mitochondrial dynamics, and neuronal survival, *BCL2* is not widely recognized as a primary genetic risk factor for ALS development While some studies have suggested a potential link between *BCL2* and ALS, the evidence remains inconclusive. As a result, *BCL2* is typically not included among the genes commonly associated with ALS (https://alsod.ac.uk, last accessed May 2023) [35]. In a 1996 study, alterations in *BCL2* and *BAX* gene expressions were found in motor neurons of ALS patients' spinal cords, with decreased *BCL2* expression compared to the control group. This suggests that changes in *BCL2* expression may be relevant to ALS progression [36]. Consequently, it is expected that the expression levels of miRNAs that interact with *BCL2* could be altered in the context of ALS. Moreover, our pathway analysis has connected *BCL2* to the ALS KEGG pathway and other relevant pathways (Fig. 2). Aberrant association of *SOD1* and *Bcl-2* was observed in ALS neurons, leading to mitochondrial aggregation and reduced ADP permeability [12, 37]. Our pathway analysis connected autophagy, apoptosis, P53 signaling, and neurodegeneration (Fig. 3, and Table 2), involving genes affecting mitochondrial dysfunction. The study also

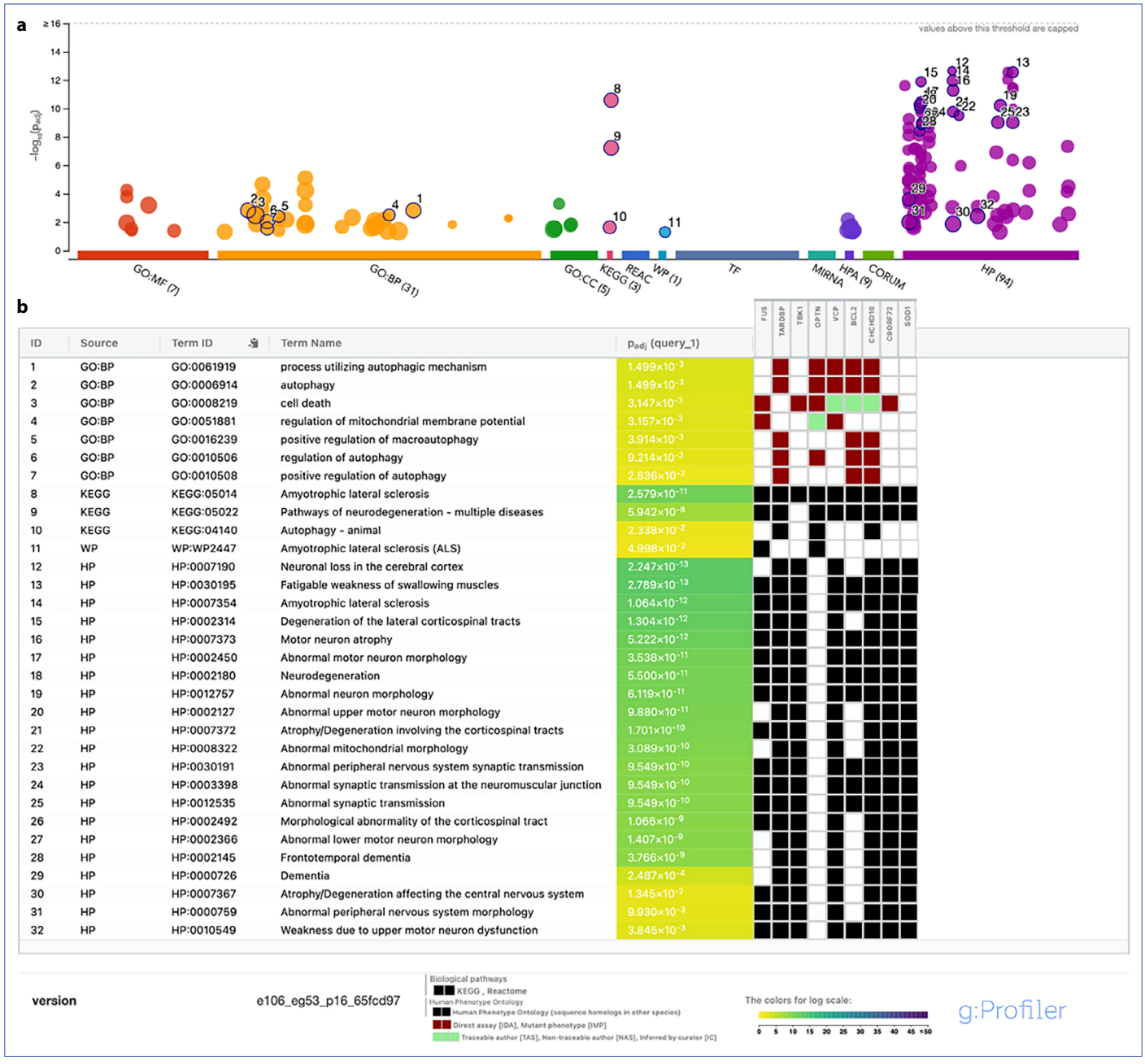

Figure 3. A visual representation of the results of g: Profiler enrichment analysis in the form of a Manhattan plot. (a) g: Profiler enrichment results. (b) A comprehensive analysis of pathways enriched in the gene list.

ALS: Amyotrophic lateral sclerosis; KEGG: Kyoto Encyclopedia of Genes and Genomes; REAC: Reactome; WP: WikiPathways; TF: Transcription factors; HPA: Human phenotype annotation; HP: Human phenotype ontology; FUS: Fused in sarcoma; TARDBP: TAR DNA-binding protein; TBK1: TANK Binding Kinase 1; OPTN: Optineurin; VCP: Valosin-containing protein; BCL2: B-cell Lymphoma 2; CHCHD10: Coiled-coil-helix-coiled-coil-helix domain-containing 10; C9ORF72: Chromosome 9 Open Reading Frame 72; SOD1: Superoxide dismutase 1.

identified specific miRNAs targeting these genes, forming a coherent network. These findings shed light on potential interactions contributing to ALS pathogenesis and offer valuable insights into the underlying molecular mechanisms.

Pathway analysis in this study supports the important roles of *TBK1* and *OPTN* in mitophagy. In ALS, mutant *OPTN* leads to mitochondrial dysfunction, causing an accumulation of mitochondria in neurons [38]. Mutant *OPTN* loses its ability to suppress NF-κB, resulting in the release of proinflammatory cytokines and neuronal cell death [39]. Mutations in *OPTN* and *TBK1* cause their accumulation in mitochondria, resulting in the production of ROS and depolarization. This accumulation also interferes with mitophagy and autophagosome formation, as shown in previous studies [40]. Abnormal mitophagy due to *TBK1* mutations contributes to the build-up of damaged mitochondria and cellular stress,

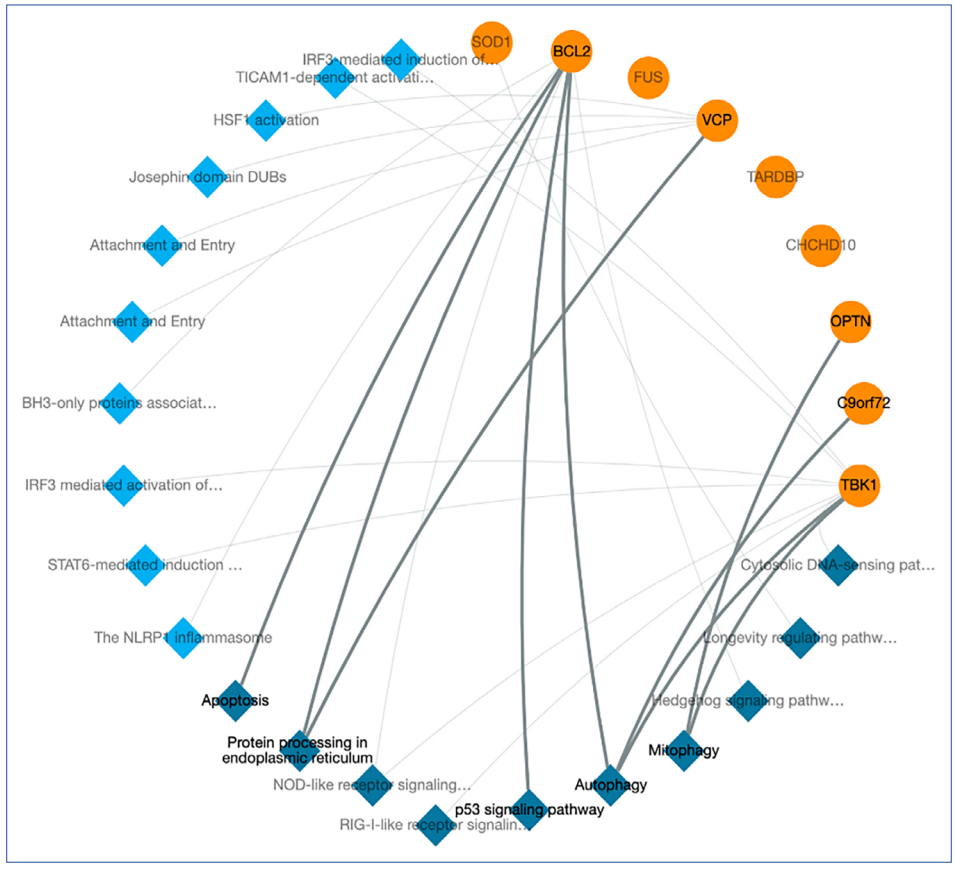

Figure 4. Pathway enrichment analysis using the CROssBAR tool.

SOD1: Superoxide dismutase 1; BCL2: B-cell Lymphoma 2; FUS: Fused in sarcoma; VCP: Valosin-containing protein; TARDBP: TAR DNA-binding protein; CHCHD10: Coiled-coil-helix-coiled-coil-helix domain-containing 10; OPTN: Optineurin; C9ORF72: Chromosome 9 Open Reading Frame 72; TBK1: TANK Binding Kinase 1; RIG-I: etinoic acid-inducible gene I; NOD: Nucleotide oligomerization domain; NLRP-1: Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-1; STAT6: Signal Transducer and Activator of Transcription 6; IRF3: Interferon Regulatory Factor 3; BH3: Pro-apoptotic members of the Bcl-2 family; HSF1: Heat shock transcription factor 1; TICAM1: TIR domain containing adaptor molecule 1; CROssBAR: Comprehensive resource of biomedical relations with knowledge graph representations (https://crossbar.kansil.org/).

potentially playing a role in ALS pathophysiology [38]. *TBK1*'s involvement in autophagy and mitophagy emphasizes its significance in the development of ALS, as supported by the pathway analyses in this study.

Recent studies have revealed that the processing of miR-125b is particularly influenced by two genes, *TARDBP* and *FUS*, both of which have been linked to ALS pathology. This suggests that miR-125b may play a significant role in the development of ALS [41]. Our investigation corroborates previous studies that have identified the *TARDBP* and *FUS* genes as targets of miR-125b, as presented in Appendix 1. In a mouse model with the *SOD1* mutation linked to ALS, miR-125b expression was observed to be increased in microglia cells . Notably, while miR-125b expression was found to be increased in microglia cells of ALS mice, it was down-regulated in ALS patients and in the NMJ of patients with sALS [42]. Furthermore, it was found to be decreased in the NMJ of patients with sALS [43]. The *in vitro* studies conducted by Giroud et al. [44] suggest that miR-125b has a regulatory function in mitochondrial biogenesis. Specifically, their experiments showed that overexpressing miR-125b-5p led to decreased mitochondrial biogenesis while knocking down miR-125b-5p resulted in increased biogenesis. Furthermore, a different study reported that miR-125b decreases mitochondrial respiration [45]. Different studies have identified *BCL2* as a target of miR-125b [46]. Our findings align with prior research indicating that the *BCL2* gene is a target of miR-125b (Appendix 1).

Variability in miRNA targets across databases may arise from differences in prediction algorithms, data sources, experimental methods, and data curation, leading to discrepancies in associated p-values. For example, in one database, the p=0.111, adjusted p=0.46895, and in another database, the p=5.98e-10 (Table 3, Row 1). These differences can be due to varying sample sizes or data comprehensiveness, impacting statistical power to detect significant miRNA-target interactions. Variations in p-values can also be influenced by specific significance thresholds used in each database, leading to differing values for the same miRNA-target interactions. During the set analysis, we identified miRNAs with low p-values associated with ALS disease across databases. Notably,

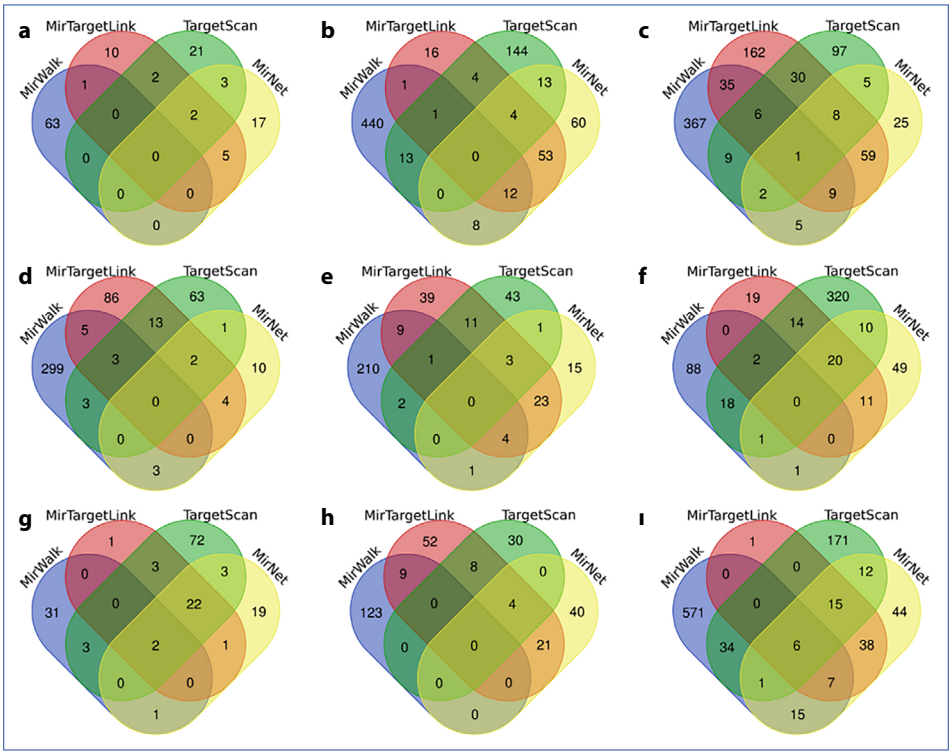

Figure 5. The Venn diagram images that illustrate the shared miRNAs from four different databases for the identified genes. (a) SOD1, (b) FUS, (c) TARDBP, (d) C9ORF72, (e) OPTN, (f) VCP, (g) CHCHD10, (h) TBK1, (ı) BCL2.

Six miRNAs targeting BCL2 were discovered to be prevalent in all four databases. One miRNA was found to target TARDBP and was common in all four databases. Two miRNAs targeting CHCHD10 were found in all four databases. SOD1, FUS, C9ORF72, TBK1, OPTN, VCP have common miRNAs in at most three databases. None of the six genes have miRNAs in common across all four databases. SOD1: Superoxide dismutase 1; FUS: Fused in sarcoma; TARDBP: TAR DNA-binding protein; C90RF72: Chromosome 9 Open Reading Frame 72; OPTN: Optineurin; VCP: Valosin-containing protein; CHCHD10: Coiled-coil-helix-coiled-coil-helix domain-containing 10; TBK1: TANK Binding Kinase 1; BCL2: B-cell Lymphoma 2.

hsa-miR-125b-5p, hsa-miR-141-3p, and hsa-miR-9-5p were among the selected miRNAs, showing statistical significance. Despite diverse p-values, these miRNAs are linked to nervous system-related mechanisms and detected in brain tissue, suggesting involvement in ALS pathogenesis (Table 3). The observed statistical significance supports their relevance as potential miRNA candidates in ALS.

Hsa-miR-125b, a member of the miRNA-125 family, has been extensively studied in various biological contexts. The research conducted by Le et al. [47] has highlighted its crucial role in promoting neuronal differentiation in human cells. MiR-125b acts as a positive regulator, facilitating the differentiation of cells into neurons by repressing multiple target genes and influencing important signaling pathways during neural development [48]. Furthermore, studies have indicated the potential involvement of hsa-miR-125b in neurodegenerative diseases. Its dysregulation has been observed in conditions such as Alzheimer's disease and Parkinson's disease, suggesting it may contribute to the disease progression by affecting genes relevant to the pathogenesis [49]. Beyond its significance in the nervous system, miR-125b also plays a role in tumorigenesis. It has been shown to target the tumor suppressor gene TP53 (p53), acting as a negative regulator of p53 expression in certain cancers, thereby promoting cell survival and proliferation. Conversely, miR-125b can act as a tumor suppressor by targeting oncogenes and inhibiting tumor growth [47]. Moreover, miR-125b has a crucial impact on the hematopoietic system. It is enriched in hematopoietic stem cells (HSCs) and plays a role in regulating HSC differentiation and lineage commitment, thereby affecting long-term hematopoietic output [50].

In our study, the miRNA set analyses revealed a significant association between miR-9 and the brain, as well as with neuronal differentiation and brain development, which is consistent with the established role of miR-9 in these processes (Table 3). Hsa-miR-9-5p is an extensively studied microRNA with significant functions in various biological processes. Coolen et al. [51] conducted research to investigate the involvement of miR-9-5p in the control of neurogenesis, the process responsible for neural stem cells differentiating into neurons. The study revealed that miR-9-5p directly targets and suppresses the expression of factors that act as regulators of neurogenesis. As a result, miR-9-5p fine-tunes the timing of neuronal differentiation, demonstrating its critical role in ensuring proper development of the nervous system. Several

Table 3. Set analyses with miRNet and TAM

miRNA function	miRNAs	p (miRNet)	Adj p (miRNet)	p (TAM)
Aging	hsa-let-7b-5p	0.111	0.46895	5.98e-10
	hsa-miR-7-5p			
	hsa-miR-181b-5p			
	hsa-miR-141-3p			
	hsa-miR-9-5p			
	hsa-miR-200c-3p			
	hsa-miR-155-5p			
	hsa-let-7b-3p			
Neuron differentiation	hsa-miR-182-5p	0.0617	0.4590538	3.89e-6
	hsa-miR-9-5p			
	hsa-miR-376c-3p			
Brain development	hsa-miR-20a-5p	0.201	0.5561	3.41e-6
	hsa-miR-103a-3p			
	hsa-miR-9-5p			
	hsa-miR-155-5p			
Oxidative stress	hsa-miR-141-3p	0.462	0.6358871	0.1808
Neuron development	hsa-miR-141-3p	0.488	0.6406563	0.1808
Apoptosis	hsa-miR-7-5p	0.623	0.7494058	1.95e-5
	hsa-miR-182-5p			
	hsa-miR-9-5p			
	hsa-miR-155-5p			
miRNA disease				
Amyotrophic lateral sclerosis	hsa-miR-125b-5p	0.00733	0.01981081	1.48e-5
	hsa-miR-141-3p			
	hsa-miR-9-5p			
miRNA tissue				
Brain	hsa-let-7b-5p	1.27e-8	5.08e-7	4.89e-4
	hsa-miR-139-5p			
	hsa-miR-7-5p			
	hsa-miR-181b-5p			
	hsa-miR-212-3p			
	hsa-miR-23b-3p			
	hsa-miR-125b-5p			
	hsa-miR-141-3p			
	hsa-miR-9-5p			
	hsa-miR-186-5p			
	hsa-miR-155-5p			
	hsa-miR-361-5p			
	hsa-miR-330-3p			
	hsa-miR-429			

TAM: Tool for miRNA set analysis (http://www.lirmed.com/tam2/); Adj: Adjusted.

studies have indicated that miR-9 could potentially regulate mature neurons and participate in neurodegeneration. Experiments in cell cultures and ALS-related model organisms have linked altered miR-9 expression to neurodegeneration [52]. Therefore, miR-9 might be a potential biomarker for ALS and warrants further investigation.

Hsa-miR-141-3p is a versatile microRNA with involvement in various biological processes, both in normal physiology and disease conditions. It has been studied in different cancer types, such as prostate, and ovarian cancers, where it can function as either a tumor suppressor or an activator depending on the context [53]. In stem cells from apical papilla (SCAPs), miR-141-3p overexpression was found to inhibit cell proliferation and accelerate cellular senescence by targeting Yes-associated protein, while inhibiting miR-141-3p promoted SCAPs' proliferative ability and delayed their senes-

cence [54]. In the context of bacterial meningitis, miR-141-3p was observed to inhibit astrocyte activation and the release of inflammatory cytokines by down-regulating high-mobility group box 1 expression [55]. In addition, miR-141-3p was shown to enhance autophagic activity when overexpressed, while low expression of miR-141-3p inhibited autophagic activity in trophoblast cells under hypoxic conditions [56]. Moreover, miR-141 was found to be upregulated in the brain in response to reactive oxygen species in Alzheimer's disease [57]. Overall, miR-141-3p exhibits diverse roles in regulating various cellular processes and responses to different pathological stimuli, making it important in understanding disease mechanisms and as a potential target for therapeutic interventions in various diseases. While our findings suggest that miR-141 may play a role in the development or progression of ALS, further research is needed to fully understand its involvement in the pathogenic mechanisms underlying ALS and other neurodegenerative diseases.

Conclusion

In this study, we conducted a comprehensive literature review to identify genes that are associated with mitochondrial dysfunction in ALS. Through *in silico* analyses, we identified three different miRNAs as potential regulators of these genes and their involvement in ALS.

While *in silico* predictions offer valuable hypotheses for potential miRNA biomarkers, this method is not without its limitations. *In silico* tools often prioritize miRNA-mRNA interactions based on sequence complementarity or conservation but may lack detailed functional annotation for the predicted targets. Consequently, false-positive or false-negative predictions can occur, and the accuracy may be affected by incomplete or biased datasets. Moreover, *in silico* tools might not fully capture the intricate regulatory networks underlying miRNA-mediated gene regulation in specific biological contexts.

In light of the constraints inherent to *in silico* methods, it is essential to undertake additional research to validate the accuracy and feasibility of the miRNAs that have been identified as potential biomarkers for the diagnosis and therapeutic intervention of ALS. Currently, we are carrying out experimental studies using blood samples from ALS patients as part of a supported project to validate the applicability of these miRNAs.

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APPENDIX 1. miRNAs targeting genes associated with mitochondrial dysfunction

miRNA	Target genes	Databases	miRNA	Target genes	Databases
hsa-miR-582-5p	*BCL2*	MirNet, TargetScan	hsa-miR-212-3p	*FUS*	MirTargetLink, MirNet
	C9ORF72	MirTargetLink, TargetScan		*TARDBP*	MirWalk, MirNet
	FUS	MirNet, TargetScan		*TBK1*	MirTargetLink, MirWalk
	SOD1	MirNet, TargetScan	hsa-miR-330-3p	*FUS*	MirNet, TargetScan
hsa-miR-141-3p	*C9ORF72*	MirTargetLink, MirWalk		*OPTN*	MirTargetLink, MirWalk
	FUS	MirTargetLink, MirNet		*TARDBP*	MirTargetLink, TargetScan
	TARDBP	MirTargetLink, MirNet	hsa-miR-376c-3p	*BCL2*	MirTargetLink, MirNet
	OPTN	MirTargetLink, MirWalk		*C9ORF72*	MirTargetLink, MirNet, TargetScan
hsa-miR-182-5p	*BCL2*	MirTargetLink, MirWalk, MirNet, TargetScan		*FUS*	MirTargetLink, TargetScan
	C9ORF72	MirTargetLink, MirNet, TargetScan	let-7b-5p	*BCL2*	MirNet, MirWalk
	TARDBP	MirNet, TargetScan		*C9ORF72*	MirNet, MirWalk
	OPTN	MirTargetLink, TargetScan		*FUS*	MirNet, MirWalk
hsa-miR-513a-5p	*SOD1*	MirTargetLink, TargetScan	hsa-miR-155-5p	*BCL2*	MirNet, TargetScan
	TARDBP	MirTargetLink, MirNet, TargetScan		*FUS*	MirNet, TargetScan
	TBK1	MirTargetLink, TargetScan		*TBK1*	MirTargetLink, mirNet, TargetScan
hsa-miR-552-3p	*SOD1*	MirTargetLink, TargetScan	hsa-miR-186-5p	*C9ORF72*	MirNet, TargetScan
	TARDBP	MirTargetLink, TargetScan		*TARDBP*	MirTargetLink, MirNet, TargetScan
	TBK1	MirTargetLink, MirWalk		*TBK1*	MirTargetLink, MirNet, TargetScan
hsa-miR-139-5p	*BCL2*	MirTargetLink, MirWalk, MirNet, TargetScan	hsa-miR-545-3p	*C9ORF72*	MirTargetLink, TargetScan
	C9ORF72	MirTargetLink, MirWalk, TargetScan		*OPTN*	MirTargetLink, TargetScan
	SOD1	MirTargetLink, MirNet, TargetScan		*TARDBP*	MirTargetLink, TargetScan
hsa-miR-125b-5p	*BCL2*	MirTargetLink, MirWalk, MirNet	hsa-miR-429	*BCL2*	MirTargetLink, MirNet
	FUS	MirTargetLink, MirNet		*TARDBP*	MirTargetLink, MirNet
	TARDBP	MirTargetLink, MirNet		*TBK1*	MirTargetLink, MirNet
hsa-miR-181b-5p	*BCL2*	MirTargetLink, MirNet	hsa-miR-200c-3p	*BCL2*	MirTargetLink, MirNet
	FUS	MirTargetLink, MirNet		*TARDBP*	MirTargetLink, MirNet
	TARDBP	MirTargetLink, MirNet		*TBK1*	MirTargetLink, MirNet
hsa-miR-9-5p	*BCL2*	MirTargetLink, MirWalk, MirNet	hsa-miR-432-5p	*BCL2*	MirWalk, TargetScan
	OPTN	MirTargetLink, MirNet, TargetScan		*OPTN*	MirTargetLink, TargetScan
	TARDBP	MirTargetLink, TargetScan		*TARDBP*	MirWalk, TargetScan
hsa-miR-20a-5p	*BCL2*	MirTargetLink, MirNet	hsa-miR-410-3p	*C9ORF72*	MirTargetLink, TargetScan
	OPTN	MirTargetLink, MirWalk		*OPTN*	MirTargetLink, TargetScan
	TARDBP	MirTargetLink, MirNet		*VCP*	MirTargetLink, TargetScan
hsa-miR-136-5p	*BCL2*	MirTargetLink, MirNet, TargetScan	hsa-miR-361-5p	*OPTN*	MirTargetLink, TargetScan
	C9ORF72	MirTargetLink, TargetScan		*SOD1*	MirNet, TargetScan
	VCP	MirTargetLink, TargetScan		*VCP*	MirTargetLink, MirNet
hsa-miR-7-5p	*BCL2*	MirTargetLink, MirWalk, MirNet, TargetScan	hsa-miR-331-3p	*FUS*	MirTargetLink, MirNet
	FUS	MirNet, TargetScan		*TARDBP*	MirWalk, MirNet, TargetScan

APPENDIX 1 (cont). miRNAs targeting genes associated with mitochondrial dysfunction

miRNA	Target genes	Databases	miRNA	Target genes	Databases
	VCP	MirTargetLink, MirNet		*VCP*	MirTargetLink, TargetScan
hsa-miR-23b-3p	*SOD1*	MirTargetLink, MirNet	hsa-miR-361-3p	*BCL2*	MirWalk, TargetScan
	TARDBP	MirTargetLink, MirNet		*TARDBP*	MirTargetLink, MirWalk, MirNet, TargetScan
	VCP	MirTargetLink, MirNet, TargetScan		*VCP*	MirTargetLink, MirNet, TargetScan
hsa-miR-103a-3p	*BCL2*	MirTargetLink, MirNet			
	TARDBP	MirTargetLink, MirNet			
	VCP	MirTargetLink, MirNet, TargetScan			

BCL2: B-cell Lymphoma 2; C9ORF72: Chromosome 9 Open Reading Frame 72; FUS: ????; SOD1: Superoxide dismutase 1; TARDBP: TAR DNA-binding protein; OPTN: Optineurin; TBK1: TANK Binding Kinase 1; VCP: Valosin-containing protein.