Neurology Asia 2026; 31(3) : 993 – 1004

Bioinformatics analysis of the alterations in neuregulin 1/4 and their correlation with ferroptosis-related genes in amyotrophic lateral sclerosis

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Abstract

Background & Objective: Dysregulation of the ErbB4 signaling pathway has been implicated as a significant factor in the pathogenesis of amyotrophic lateral sclerosis （ALS）, with neuregulin 1 (NRG1) and NRG4 serving as key regulatory ligands of ErbB4. This study mainly employs bioinformatics approaches to investigate the association between changes in ferroptosis-related genes and NRG1/4 in ALS. *Methods:* Differentially expressed genes (DEGs) in ALS spinal cord tissue were identified from the GEO database, with a focus on ferroptosis-related genes sourced from FerrDb. Subsequent protein-protein interaction (PPI) network was constructed using Cytoscape software. The expression levels of NRG1 and NRG4 in ALS were then examined, followed by a correlation analysis between these genes and main ferroptosis-related DEGs in ALS. *Result:* In the anterior horn tissue, 6 ferroptosis-related genes were downregulated and 3 were upregulated, whereas in motor neurons, 11 ferroptosis-related genes were upregulated and 8 were downregulated. In normal anterior horn tissues, NRG4 exhibits a positive correlation with MAP3K14, whereas in anterior horn tissues of ALS patients, it is positively correlated with PANX2. In motor neurons, NRG1 was negatively correlated with JUN, SLC7A11, and PANX2 in ALS. Additionally, NRG4 was negatively correlated with TIMP1, SOX2, and AR in normal controls, and with CFL1 and JUN in ALS. PANX2 showed a positive correlation with both NRG1 and NRG4 in both normal controls and ALS patients.
Conclusion: The bioinformatics analysis results underscore the potential of NRG1/4 signaling in mitigating ALS by regulating specific ferroptosis-related genes.

Keywords: Amyotrophic lateral sclerosis (ALS), spinal cord, bioinformatics, ferroptosis, neuregulins, ErbB4.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the degenerative loss of upper and lower motor neurons in the brain and spinal cord.[1] With the in-depth research on ALS, numerous pathogenic genes have been screened, such as TDP-43[2], SOD1[3], ErbB4[4], FUS[5], etc. Mutations in ALS-related pathogenic genes result in the abnormal accumulation of proteins within mitochondria, leading to mitochondrial depolarization and the excessive production of reactive oxygen species (ROS), thereby inducing oxidative stress and subsequent cell death, including ferroptosis.[6]

Ferroptosis is biochemically marked by the accumulation of lipid-based ROS.[7] The disruption of iron homeostasis in brain tissue can lead to a significant production of ROS in neuronal cells, ultimately resulting in severe oxidative damage to vulnerable subcellular structures.[8] Inhibiting neuronal ferroptosis has been reported to effectively enhance the prognosis of AD and PD patients.[6]

Neuregulins (NRGs) are integral members of the epidermal growth factor (EGF)-like extracellular ligand family and serve as ligands for the ErbB family of receptor tyrosine kinases.[9] NRGs are fundamentally involved in

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Date of Submission: 4 May 2025; Date of Acceptance: 6 March 2026

https://doi.org/10.54029/2026hhj

regulating cellular proliferation[10], differentiation and migration of neural crest cells.[11] They also function in the survival and maturation of astrocytes, as well as in the growth and differentiation of epithelial, glial, and muscle cells.[12] Some studies have identified loss-of-function mutations in NRG receptor ErbB4 as a causative factor for ALS in the $SOD1^{G93A}$ mouse model.[13] Overexpression of NRG1-III improves locomotor performance and reduce gliosis in female $SOD1^{G93A}$ mice. NRG4 typically exerts its functions on target cells through autocrine, paracrine, or juxtacrine mechanisms.[14] Moreover, NRG4 has been identified as a principal regulatory factor influencing dendritic growth and development of pyramidal neurons in the developing mouse neocortex.[15] Research has also demonstrated a reduction in dendritic structure and somatic cell size in NRG4 knockout pyramidal neurons within the neocortex of developing mice.[16]

In this research, we employed bioinformatics methodologies to analyze differentially expressed genes (DEGs) in spinal cord tissue samples from ALS patients. Furthermore, we examined the differential expression of NRG1/4 between normal and ALS conditions and assessed the correlation between NRG1/4 and the principal identified ferroptosis-related genes. The primary objective of this study is to elucidate the mechanisms underlying neuronal ferroptosis in ALS, with a particular emphasis on the interplay between ferroptosis and NRG pathways, thereby identifying potential diagnostic and therapeutic targets for ALS.

METHODS

Screening of differential genes related to ferroptosis in ALS

The comprehensive experimental procedure is illustrated in Figure 1. Microarray datasets GSE18920, derived from the spinal cord of ALS patients, were obtained from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Ustilizing the annotation information retrieved from the GEO platform (GPL), probe ID information was subsequently converted into gene markers during the gene expression analysis. In addition, a total of 388 ferroptosis-related genes were retrieved from the FerrdDb database (http://www.zhounan.org/ferrdb), compassing driver genes, suppressor genes, and gene markers, among others.

The non-coding and visual online analysis tool, Clinical Bioinformatics Home (available at www.aclbi.com), was utilized to conduct differential gene expression analysis. DEGs were screened with fold change (FC) ≥ 1.3 and p-value < 0.05. High-definition vector graphs depicting the DEGs, along with the original data in tabular format, were simultaneously downloaded. The intersection of the sets of DEGs and ferroptosis-related genes was performed to identify the subset of DEGs associated with ferroptosis in both anterior horn and motor neurons of the spinal cord in both the normal controls and ALS patients.

Expression of NRG1 and NRG4 in the spinal cord under pathological conditions of ALS

From this subset, NRG1 and NRG4 were selected for further analysis. The relative levels of NRG1 and NRG4 were subsequently visualized in the anterior horn and motor neurons of the spinal cord in both normal controls and ALS patients.

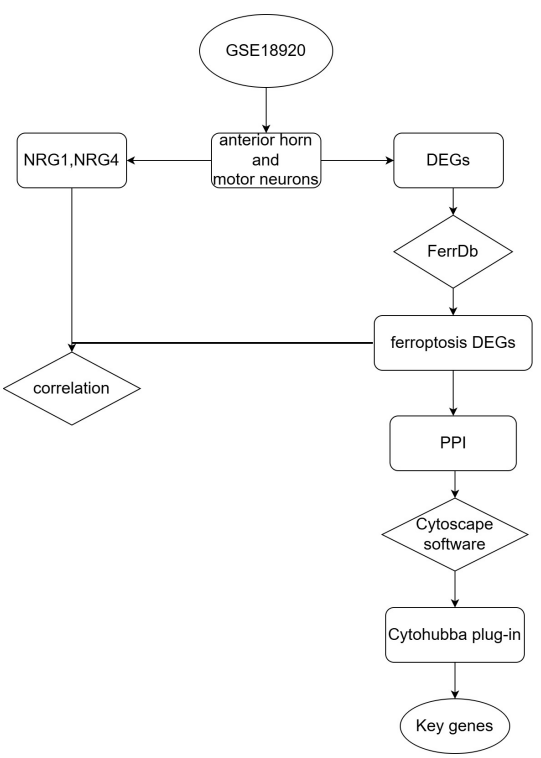

Figure 1. Flowchart for the overall bioinformatics analysis.

Correlation analysis between NRG1 and NRG4 and differentially expressed genes of ferroptosis

We utilized the online bioinformatics analysis platform provided by Xiantao Academic (https://www.xiantaozi.com) to perform gene correlation analysis. Initially, we compiled a dataset from the downloaded information and subsequently employed the batch correlation analysis tool available on the website to generate a correlation table. Following this, we utilized the correlation mapping tool to produce correlation scatter plots. Spearman correlation analysis was conducted to assess the relationship between NRG1 and NRG4 and main ferroptosis-related genes in both normal controls and ALS patients. A p-value of less than 0.05 was considered indicative of statistical significance.

Construction and screening of differentially key genes in the protein-protein interaction network (PPI)

Initially, we employed the GeneMANIA database (http://genemania.org/) to integrate differential genes into the construction of PPI networks. Subsequently, genes differentially associated with ferroptosis were input into the STRING database (https://cn.string-db.org/), specifying the species as Homo sapiens, and a TSV file was exported. The STRING database is instrumental in identifying protein interactions. The exported file from the STRING database was then analyzed for protein interaction networks using Cytoscape software. Key genes were identified using the Cytohubba plug-in within the software, applying MCC（Maximal Clique Centrality）algorithms.

RESULTS

Differential analysis

Dataset GSE18920, which includes 10 control and 12 sporadic ALS lumbar spinal cord specimens, was utilized for differential analysis. We identified a total of 320 DEGs from the anterior horn of the spinal cord under ALS. Volcanic and heatmap analyses illustrated the expression profiles and distribution patterns of these DEGs (Figure 2A and B), comprising 73 upregulated and 247 downregulated genes. In the mortor neurons, we detected a total of 667 DEGs. The corresponding volcano and heat map analyses depicted the expression and distribution of these DEGs (Figure 2C and D), which include 274 upregulated genes and 393 downregulated genes.

Differentially expressed genes associated with ferroptosis

The ferroptosis-related genes identified from the FerrDb database were cross-referenced with the DEGs from the GSE18920 dataset. This cross-referencing identified four downregulated DEGs associated with ferroptosis suppression in the anterior horn tissue, including CREB3, TP63, PANX2, MAPKAP1. Additionally, five DEGs related to ferroptosis driver genes were identified in the anterior horn tissue. Among them, TIMP1, ZEB1, and DCAF7 are up-regulated, while MAP3K14 and BID are downregulated. In the motor neurons, 10 DEGs related to ferroptosis suppression were identified, with seven upregulated and three downregulated. Furthermore, the study identified 9 ferroptosis driver genes in the motor neurons, with four genes upregulated and five downregulated (see Table 1).

Relative levels of NRG1 and NRG4 in the spinal cord in ALS

Within the Clinical Bioinformatics platform, specimens were categorized into the control and ALS groups, each of which containing the anterior horn and motor neurons. Using the gene expression comparison module, the control and ALS groups were designated as sample group 1 and sample group 2 respectively, and NRG1 selected as the gene of interest. This analysis generated visual expression dot plots for the anteriror horn (Figure 3A) and motor neurons (Figure 3B). The results indicated a significant reduction in relaive NRG1 levels in the spinal cord of ALS patients for both the anterior horn ($p = 0.0079$) and motor neurons ($p = 0.0050$) (Figure 3A, B). Similarly, the control and ALS groups were assigned to sample group 1 and sample group 2 respectively for the analysis of NRG4 expression. NRG4 was selected as the gene of interest, resulting in the generation of visualized expression dot plots for the anterior horn (Figure 3C) and motor neurons (Figure 3D). The analysis demonstrated a significantly reduced relative NRG4 level in the anterior horn of ALS group ($p = 0.0046$) (Figure 3C).

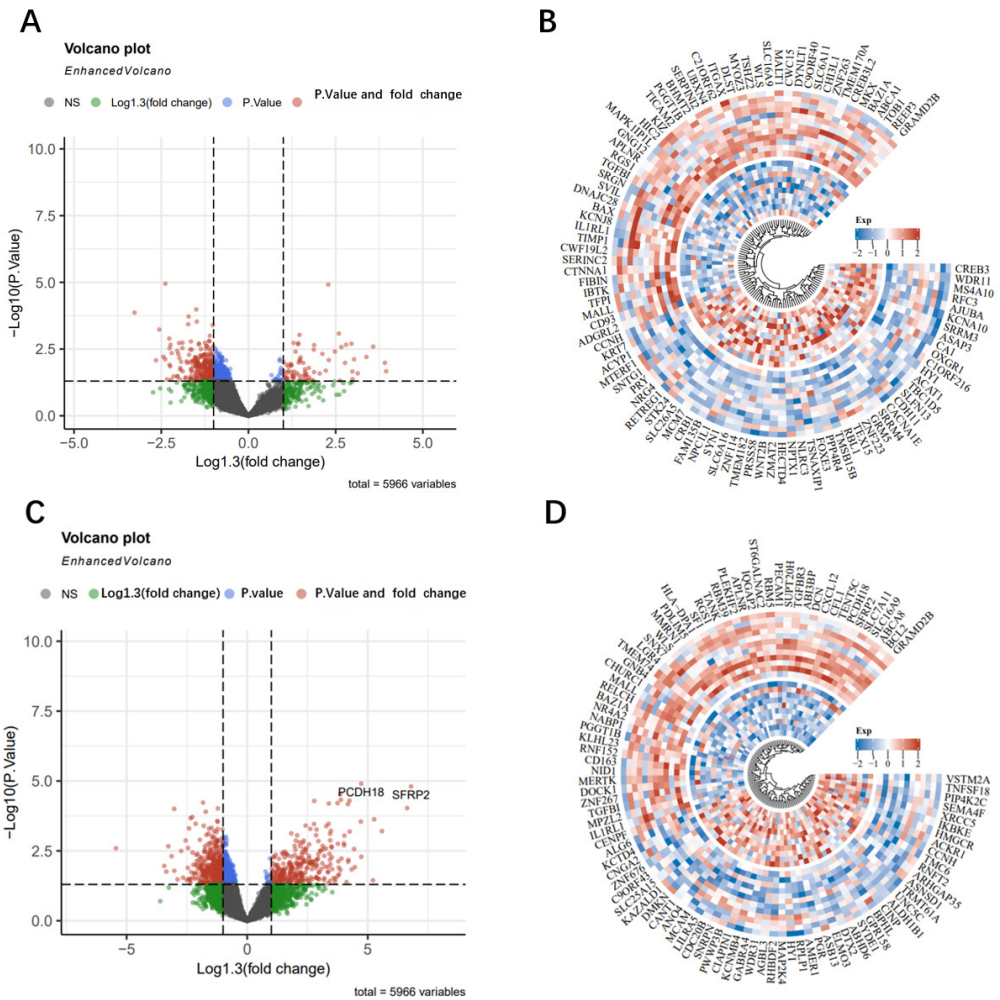

Figure 2. Heat maps and volcano plots of DEGs in the anterior horn and motor neurons of the spinal cord from both normal controls and ALS patients. The volcano plot (A) and heat map (B) of DEGs in the anterior horn. The volcano plot (C) and heat map (D) of DEGs in the motor neurons. Gene expression levels are color-coded, with red indicating high expression and blue indicating low expression.

Correlation analysis of NRG1 and NRG4 with differentially expressed ferroptosis-related Genes

To identify the role of NRG1 and NRG4 in ferroptosis regulation in ASL, we utilized Xiantao Academic Online Bioinformatics Analysis Tool to explore the correlations between NRG1, NRG4 and some differentially expressed ferroptosis-related genes.

In the anterior horn of normal control, a weak positive correlation was observed between NRG4 and the ferroptosis-suppresor gene PANX2. In contrast, in the anterior horn of ALS patients, NRG4 and PANX2 exhibited a moderate positive correlation ($p < 0.05$) (Figure 4). Additionally, in the normal anterior horn, NRG4 and the ferroptosis-driver gene MAP3K14 exhibited a significantly moderate positive correlation ($p < 0.05$). Nevertheless, in the anterior horn of ALS patients, a weak negative correlation was observed between NRG4 and MAP3K14 (Figure 4).

In the motor neurons of ALS patients, both NRG1 and NRG4 demonstrated a moderate negative correlation with the ferroptosis-suppresor gene JUN ($p < 0.05$) (Figure 5A). In the motor neurons of normal controls, NRG4 and the ferroptosis-suppresor gene AR exhibited a strong negative correlation ($p <$

Table 1: Differentially expressed ferroptosis suppressor and driver genes in ALS compared to normal control

expression level	Suppressor		Driver	
	anterior horn	motor neurons	anterior horn	motor neurons
Down	CREB3	AR	MAP3K14	PTPN6
	TP63	PML	BID	LIG3
	PANX2	PANX2		PPARG
	MAPKAP1			IDO1
				GLS2
Up		SLC7A11	TIMP1	TIMP1
		SOX2	ZEB1	TF
		LAMP2	DCAF7	ATM
		FTL		CFL1
		PARP8		
		TF		
		JUN		

0.05). However, in the motor neurons of ALS, a weak positive correlation was observed between them (Figure 5B). Additionally, both NRG1 and NRG4 demonstrated a moderate positive correlation with PANX2 in the motor neurons of both normal controls and ALS patients (Figure 5C). Intriguingly, both NRG1 and NRG4 demonstrated a negative correlation with the

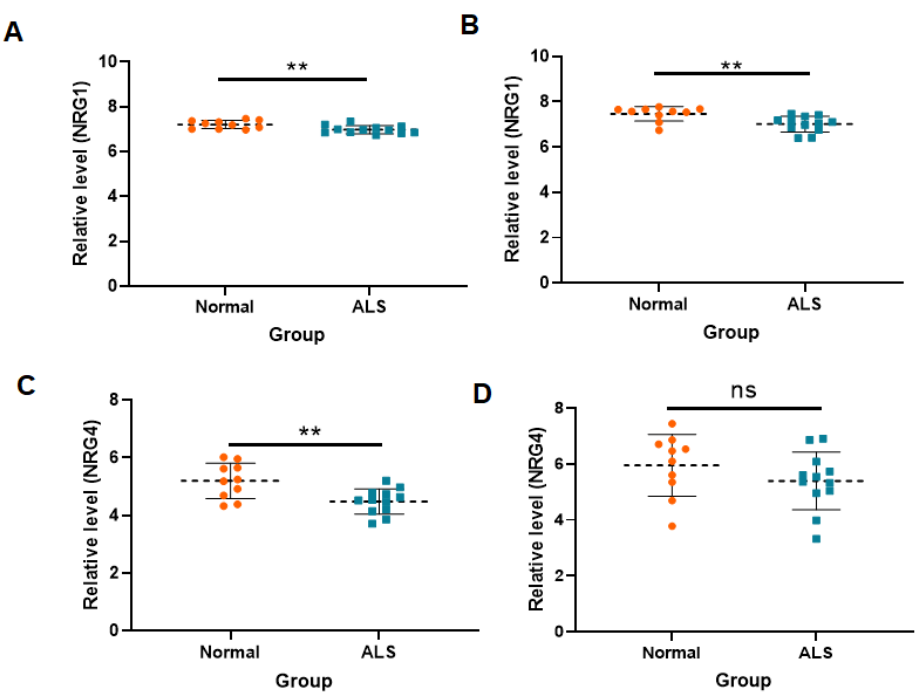

Figure 3. Dot plots depicting the relative levels of NRG1 and NRG4.
The visualized dot plots of NRG1in the anterior horn (A) and motor neurons (B) from normal control and ALS groups. The visualized dot plots of NRG4 in the anterior horn (C) and motor neurons (D) in normal control and ALS groups. Data are expressed as means ± SD, and unpaired t-test was performed. n = 10 for normal control, and n = 12 for ALS. **p < 0.01; ns, not significant.

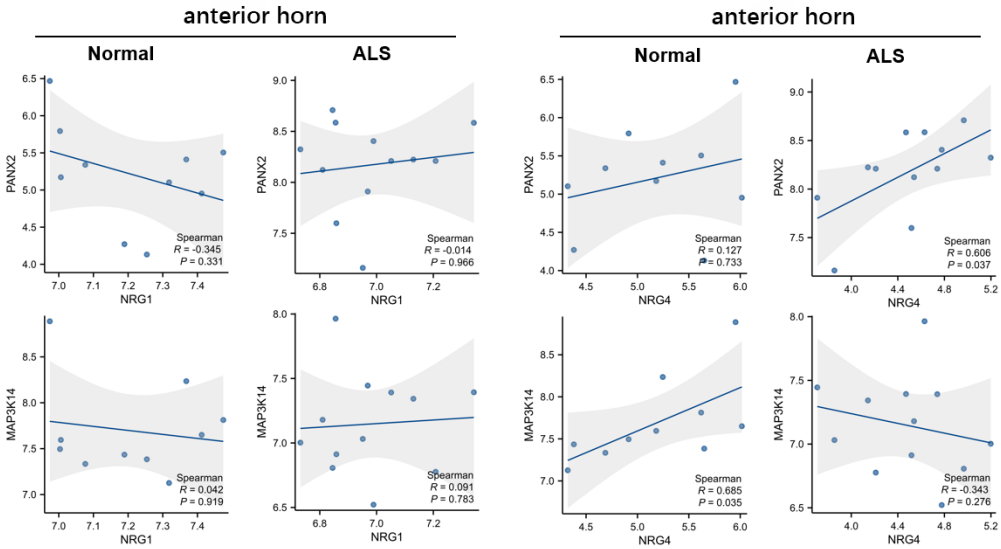

Figure 4. Scatter plots depicting the correlation between NRG1 and NRG4 and ferroptosis-related DEGs in the anterior horn of both the normal and the ALS groups.
Scatter plots showing the correlation between NRG1, NRG4 and either PANX2 or MAP3K14 in both normal controls and ALS patients. R, the correlation coefficient. The absolute value of the correlation coefficient indicates the degree of correlation, with 0-0.3 indicating weak or no correlation, 0.3-0.5 indicating weak correlation, and 0.5-0.8 indicating moderate correlation; 0.8-1 represents strong correlation.

ferroptosis-suppresor gene SLC7A11 in both normal controls and ALS samples. Significance was observed in the correlation between NRG1 and SLC7A11 in the motor neurons of ALS patients ($p < 0.05$) (Figure 5D). Furthermore, both NRG1 and NRG4 demonstrated a negative correlation with the ferroptosis-suppresor gene SOX2 in the motor neurons of both normal controls and ALS patients, with a statistical significance detected in the normal control ($p < 0.05$) (Figure 5E).

In the motor neurons of ALS, NRG1 and the ferroptosis-driver gene CFL1 demonstrated a moderate negative correlation, whereas NRG4 and CFL1 showed a moderate negative correlation in both normal and ALS motor neurons ($p < 0.05$) (Figure 5F). NRG1 exhibited a moderate negative correlation with the ferroptosis-driver gene TF in normal motor neurons ($p < 0.05$), and both NRG1 and NRG4 showed a weak positive correlation with TF in ALS samples (Figure 5G). Additionally, both NRG1 and NRG4 displayed a negative correlation with the ferroptosis-driver gene TIMP1 in the motor neurons of both normal controls and ALS patients (Figure 5H).

PPI network analysis of DEGs related to ferroptosis and identification of key genes in motor neurons

Initially, the GeneMANIA database was employed to import DEGs associated with ferroptosis. Through this process, predicted outer-layer genes of the PPI network were obtained. These genes are potentially involved in the same pathways or complexes as the ferroptosis-related DEGs and may exhibit co-expression or similar enzymatic functions (Figure 6A). Subsequently, the corresponding data file was downloaded. Following this, the STRING database was utilized to import the genes, specifying Homo sapiens as the species, and the data was exported as a TSV file. The CytoHubba plugin within Cytoscape 3.8.2 was then used to run the MCC algorithm, identifying the top ten genes with the highest scores (Figure 6B). Additionally, the Degree algorithm was applied to determine the top ten genes. From these, the DEGs related to ferroptosis were selected as key genes. Ranking from 1 to 10, they are JUN, PPARG, SOX2, ATM, AR, ZEB1, CFL1, PML, SLC7A11, and TIMP1 in sequence.

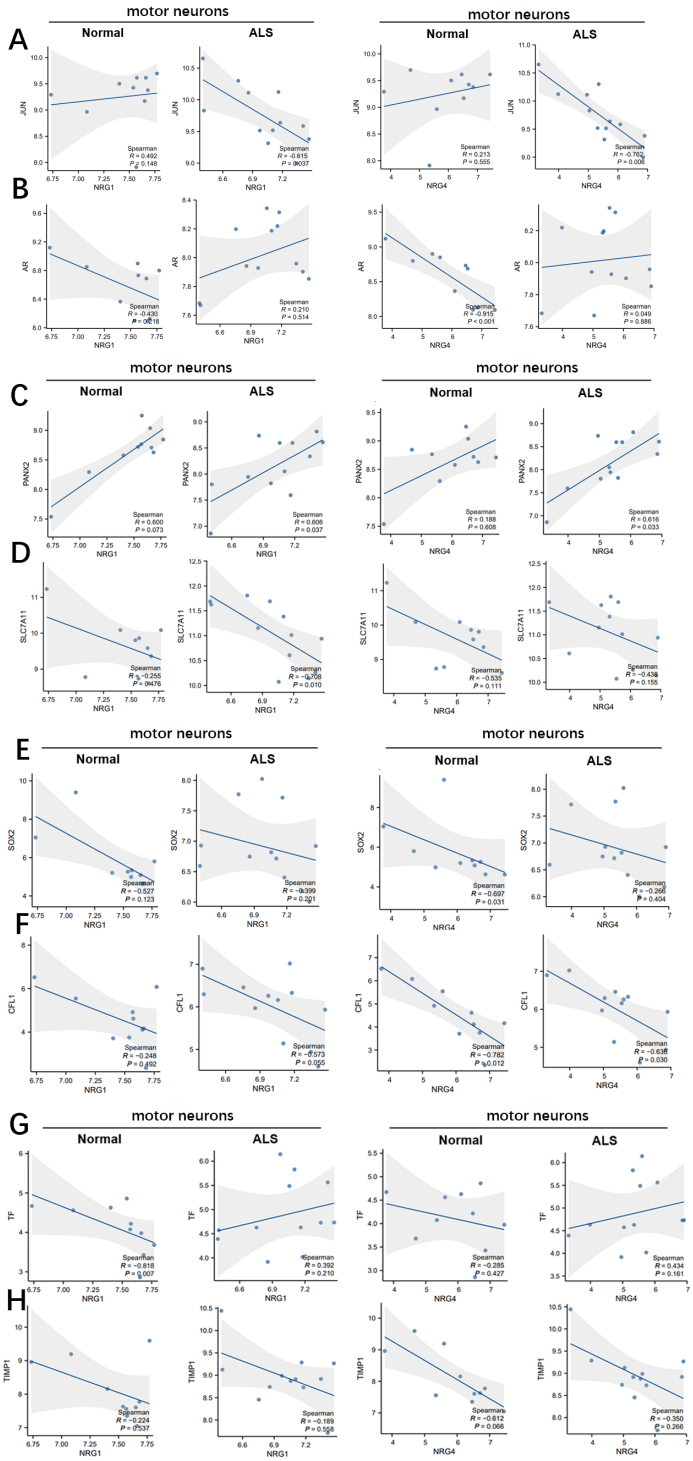

Figure 5. Scatter plots depicting the correlation between NRG1 and NRG4 and ferroptosis-related DEGs in both normal controls and ALS patients.

Scatter plots showing the correlation between NRG1 and NRG4 with JUN (Fig. 5A), AR (Fig. 5B，PANX2 (Fig. 5C), SLC7A11 (Fig. 5D), SOX2 (Fig. 5E), CFL1 (Fig. 5F), TF (Fig. 5G), and TIMP1 (Fig. 5H), in both normal control and ALS patients. R, the correlation coefficient. The absolute value of the correlation coefficient represents the degree of correlation, with 0-0.3 indicating weak or no correlation, 0.3-0.5 indicating weak correlation, and 0.5-0.8 indicating moderate correlation; 0.8-1 represents strong correlation.

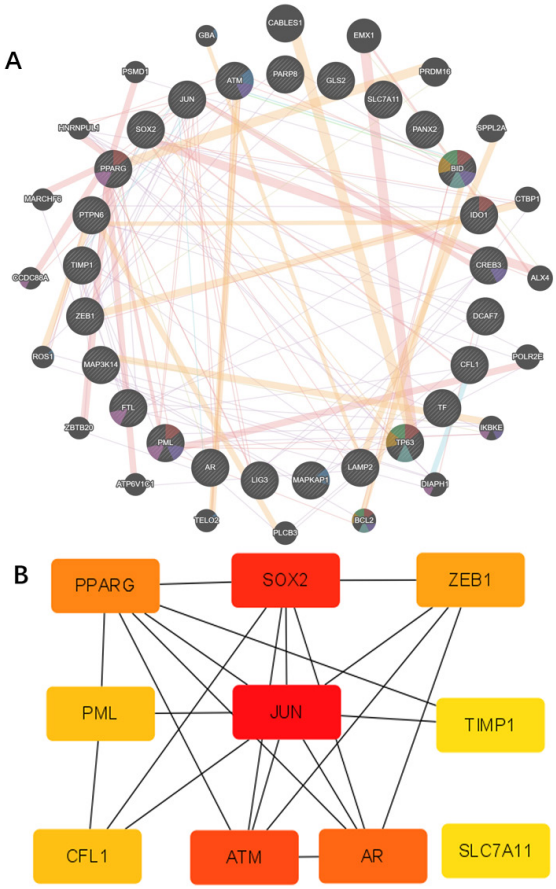

Figure 6. PPI network of DEGs related to ferroptosis in motor neurons of ALS and PPI network for the top hub genes.
(A) The PPI network, with predictive outer layer-genes, participates in the same pathway or complex as the ferroptosis-related DEGs, and may exhibit co-expression or similar enzyme functions. The inner layer represents the interaction among differentially expressed ferroptosis-related genes. (B) This PPI employs the CytoHubba plugin in Cytoscape 3.8.2 to execute the MCC algorithm, thereby identifying the top genes with the highest scores.

DISCUSSION

In this study, we identified ten genes associated with ferroptosis, namely JUN, PPARG, SOX2, ATM, AR, ZEB1, CFL1, PML, SLC7A11, and TIMP1, as key genetic elements in ALS.

JUN is the major component protein of activated protein-1 (AP-1), including c-Jun, JunB and JunD. The c-Jun N-terminal kinase (JNK) can act as a stress activated protein kinase, inducing cell apoptosis upon sustained activation.[17,18] A study has revealed that downregulating JNK signaling exerts a protective effect on neurodegenerative diseases.[19] Meanwhile, JNK promotes endoplasmic reticulum (ER) stress, causing SOD1 mutation-induced neuronal death in ALS mice and playing a role in the pathology of ALS.[20] This is consistent with the results of our research. These findings imply that JUN and NRG1 exhibit opposing physiological functions, indicating a negative correlation between them in motor neurons of ALS patients. Similarly, NRG4 plays a positive role in maintaining the complexity and morphology of neuronal dendrites.[21] This evidence suggests that NRG4 and JUN may have opposite effects in neurons, and their negative correlation in the motor neurons of ALS patients appears to corroborate this.

The maintenance of the undifferentiated state of neural stem cell is primarily associated with SOX2 expression[22,23], which inhibits neuronal differentiation.[24] During the process of neuronal differentiation, SOX2 expression

is downregulated.[25] Additionally, research indicates that SOX2 binds to the promoter region of SLC7A11, thereby reducing its expression and increasing the susceptibility of cancer cells to ferroptosis.[26] Our research results indicate that there is a substantial negative correlation between NRG4 and SOX2 in the motor neurons of the normal group. However, such a correlation is absent in the ALS group. Therefore, the modulation of SOX2 by NRG4 may contribute to stabilizing the size and complexity of dendritic spines in normal motor neurons under normal conditions.[21]

The overexpression of SLC7A11 has been implicated in specific neurodegenerative alterations.[27,28] The elevation of SLC7A11 levels can improve cognitive deficits in AD. However, studies also indicate elevated expression of SLC7A11 and increased lipid peroxidation in AD.[29] This evidence suggests that SLC7A11 may play a bidirectional regulatory role in neurodegenerative diseases. In ALS mice with mutated SOD1, elevated expression of SLC7A11 provided neuroprotection for motor neurons and alleviated the pathological progression of ALS.[30] Conversely, the inhibition of NRG1 has been shown to exacerbate ALS progression.[31] In the context of ALS, the observed negative correlation between NRG1 and SLC7A11 expression in motor neurons can be ascribed to the combined effects of NRG1 downregulation and increased SLC7A11 expression. Both of these factors potentially contribute to ALS progression.

Studies have demonstrated that an elevation in TIMP1 can protect neurons by suppressing microglial activation.[32] However, we observed a negative correlation between NRG4 levels and TIMP1 in normal motor neurons. In contrast, this correlation was weakened in motor neurons of ALS patients, ruling out the potential interaction between them during ALS. The functional impairment of AR can increase the vulnerability of motor neurons.[33] In addition, the abnormal function of AR is one of the clarified mechanisms of spinal cord and bulbous muscle atrophy (SBMA).[34] NRG1 has been identified as neuroprotective in the context of ALS.[35] Our study observed a non-significant negative correlation between NRG1 and AR in normal, and a weak non-significant positive trend in ALS motor neuron tissues, indicating altered AR-NRG1 functional crosstalk during ALS pathogenesis. The decrease in NRG4 expression results in a reduction in the size and complexity of neuronal dendrites.[21] A negative correlation was detected between AR and NRG4 expression in motor neurons from the control group, which raises the hypothesis that NRG4 exerts regulatory effects opposing those of AR at the physiological level.

Cofilin 1 (CFL1) is the main subtype of Cofilin expressed in the brain. Overexpression of CFL1 can cause neuronal death and exacerbate dopaminergic neuron damage and motor dysfunction, thereby promoting the progression of PD.[36] CFL1 expression is dysregulated in AD, manifested as synaptic defects and neuronal dysfunction.[37] NRG1 has a neuroprotective role, safeguarding neurons against glutamate-induced cytotoxicity.[38] The maintenance of NRG4 levels has a positive significance for maintaining the complexity and area of neuronal dendrites.[21] CFL1 may have opposite effects to NRG1 and NRG4, as evidenced by the negative correlation observed between CFL1 and NRG1, NRG4 in the motor neurons of ALS patients.

Decreased expression of PANX2 promotes the differentiation of immature neurons, while its re-expression in mature neurons promotes the localization of presynaptic neurons to active sites of development.[39] While NRG4 has a positive effect on dendritic size and complexity as well as neuronal area[21], PANX2 and NRG4 may have similar effects, as evidenced by the positive correlation observed between them in anterior horn and motor neurons of ALS patients in our study.

MAP3K14 is a key signaling component of the non-canonical (nc) NF-κB pathway. The ncNF-κB pathway has been shown to play a crucial role in muscle tissue. Its overactivation can lead to muscle atrophy[40], while its inhibition can reduce the loss of motor neurons.[41] Similarly, in ALS mice, the inhibition of NF-κB can prolong mouse lifespan and reduce the level of mutated SOD1.[42] However, NF-κB also involves in the synapse formation[43], and the inhibition of NF-κB pathway can lead to a decrease in the size and density of dendrites.[44] Studies have shown that the inhibition of ncNF-κB can improve motor function in ALS mouse models. Nevertheless, the absence of the ncNF-κB can result in increased inflammatory infiltration toxicity of mutated SOD1.[45] We observed a significant positive correlation between NRG4 and MAP3K14 in the anterior horn of the healthy control group, whereas such a correlation does not exist in ALS patients. This implies that their interaction may play differential roles under normal and ALS conditions.

TF binds with iron to form transferrin-bound iron (TBI), which is a relatively stable form of iron. The divalent metal transporter DMT1 mainly transports non transferrin bound iron (NTBI).[46] Studies have shown that upregulation of transferrin receptor 1 (TFR1) and DMT1 expression in ALS mice results in an increase in free iron, which promotes lipid peroxidation and subsequently leads to ferroptosis.[47] Consequently, we observed a strongly negative correlation between TF and NRG1 in normal motor neurons, suggesting that the downregulation of NRG1 and the upregulation of TF are contributing factors in the pathogenesis of ALS.

In conclusion, this study offers critical reference data for investigating the pathological mechanisms of ferroptosis in ALS and establishes a foundational basis for research on ferroptosis-related genes that require further validation in degenerative neurological diseases. Furthermore, this study analyzed the correlation between ferroptosis and NRGs, and verified the changes of NRGs in ALS. Our recent reports have demonstrated that an NRG1-mimicking small molecule can alleviate ferroptosis of senescent neurons[48], as well as improve neuronal and memory deficits in APP/PS1 transgenic mice and mice exposed to polystyrene microplastics.[49,50] Thus, the present data provide further novel information for the treatment of ALS by interfering with ferroptosis through the NRG-ErbB4 signaling pathway.

ACKNOWLEDGEMENTS

We would like to first thank the reviewers for their constructive comments and suggestions, which have helped improve the quality of our manuscript. Secondly, we would like to thank Ouyang Song for his assistance in formatting the article.

DISCLOSURE

Availability of data：The dataset generated and analyzed in the current research process can be provided according to the reasonable requirements of the corresponding author

Conflict of interest: None

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