



Gene Network Visualization and Functional Enrichment Analysis in Genes Associated with Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a fatal, progressive neurodegenerative disease. There are several genes associated with ALS such as SOD1, TARDBP, OPTN, VCP, UBQLN2 and C9orf72. For this study a variety of tools were used in order to 1) identify genes linked with ALS (Ensembl), 2) create a gene network (GeneMANIA), 3) perform functional analysis (Cytoscape), 4) create a heat map and ascertain co-expression levels between genes (STRING) and 5) to detect overlapping genes between several databases (Ensembl, DisGeNet and UniProt). Numerous key genes and pathways were identified that could play a role in the pathogenesis of ALS. However, additional examination is needed in order to establish the exact mechanism of action of these genes and pathways.

Keywords

Amyotrophic Lateral Sclerosis (ALS); Neurodegenerative diseases; Gene networks; Gene network visualization; Cytoscape

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease or more specifically Motor Neuron Disease (MND) and it is characterized by muscle weakness, atrophy and loss of upper and lower motor neurons [1]. Even though it belongs to the neurodegenerative category, it poses distinct difficulties to patients; caregivers as well as healthcare professionals seeing as it significantly differ from other neurodegenerative diseases like Alzheimer's disease or Parkinson's disease. This is mainly connected with the age of the affected patients which is usually lower in ALS cases than that of other neurodegenerative diseases as well as the prognosis and progression of ALS. Since there is no cure for the disease, healthcare professionals primarily aim to manage the symptoms of ALS patients. ALS is a rapidly progressive disease. At the onset of the disease, the majority of the patients (around 40-60%) experience muscle weakness in the upper extremities and approximately 20% in the legs [2]. As the disease advances, more progressive symptoms of muscle atrophy appear [2]. The degree of atrophy can also be used as an approach to monitor functional impairment which can predict survival time and aid health care professionals to provide the best possible therapeutic plan for the patient [3]. ALS is a very complex neurodegenerative disease that is believed to involve multifactorial mechanisms of action that impact neurodegeneration. However, these mechanisms still remain unclear. A proposed mechanism of ALS pathogenesis is as follows: impaired glutamate uptake by astrocytes results in an upsurge at glutamate excitotoxicity. Increased neurotransmitter glutamate in the synaptic cleft results in increased inflow of Ca²⁺ ions in the neurons. The amplified Ca²⁺ ions levels would normally be removed by mitochondria. However, due to mitochondrial dysfunction they remain in the cytoplasm. Subsequently, the activation of Ca²⁺ dependent enzymatic pathways which contributes to oxidative stress results to neuro degeneration [4]. The majority of ALS cases, 90-95%, are sporadic meaning that are not hereditary. The rest of the cases around 5- 10% are familial and the result of gene mutations. Established risk factors for ALS include: 1. Heredity, 2. Age, 3. Sex, 4. Genetics and 5. Several environmental factors (e.g. smoking). Gene mutations linked with ALS are most commonly found in SOD1, TARDBP, OPTN, VCP, UBQLN2 and C9orf72 and form intercellular aggregates, increase of oxidative stress and contribute to the impairment of axonal transport [4]. However, several other genes are associated with the disease. In the present study genes associated with ALS were analyzed in terms of functional enrichment and co-expression. Gene co-expression networks are most commonly used to correlate genes with biological processes, molecular functions and cellular components.

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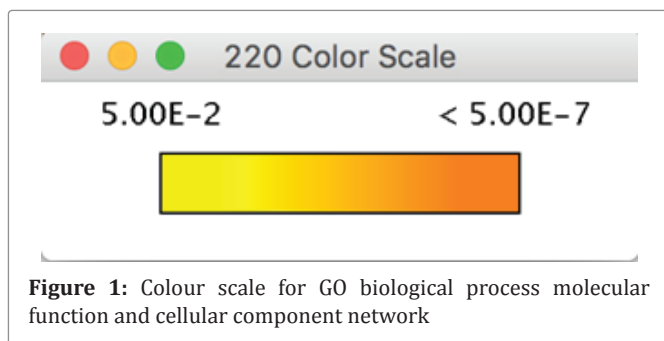


Figure 1: Colour scale for GO biological process molecular function and cellular component network

Edge	Colour
Physical interactions	Red
Co-expression	Pink
Predicted gene associations	Orange
Co-localization	Purple
Genetic interactions	Green
Pathway commonalities	Blue
Shared protein domains	Yellow

Table 1: Visualization parameters for the gene network

Methods

Gene Identification

In order to identify the majority if not all the genes associated with ALS, the database known as Ensembl was utilized. Ensembl is a genome database joint scientific project between the European Bioinformatics Institute and the Wellcome Trust Sanger Institute (<https://www.ensembl.org/index.html>). Ensembl is a genome browser that retrieves genomic information offering scientists a centralized resource tool. The search comprised of the term “Amyotrophic Lateral Sclerosis” in Homo Sapiens (organism of choice). The results summary provided 1087 loci/genes associated with ALS. After removing the duplicates, the final gene count associated with ALS was 245. In the Ensembl results table, some genes appeared to be linked with dementia and not ALS. Removal of these genes resulted in the final list of 220 genes linked with ALS.

Visualisation of gene interaction network

The list of the 220 genes related with ALS as provided by the ENSEMBL database, was uploaded to the GeneMANIA web tool (<http://genemania.org>). GeneMANIA is a website that provided with a given query gene list may that be single gene queries; multiple gene queries or network search delivers to the user the most closely connected genes amongst the networks and attributes. It recognizes co-expression, co-localization, pathway interactions as well as genetic and physical interactions in the gene network [5]. It indexes 2,277 association networks and contain 597,392,998 interactions obtained from 163,599 genes origination from 9 organisms (data obtained from the GeneMANIA website). A gene network provides visualization of interactions between a set of genes, where each gene is a node and their connections are represented by edges which characterize the functional associations between the genes. The edges between the nodes represent: 1. Physical interactions in red, 2. Co-expression in pink, 3. Predicted gene associations in orange, 4. Co-localization in purple, 5. Genetic interactions in green, 6. Pathway commonalities in blue and 7. Shared protein domains in yellow. The visualization parameters of the edges in Figure 2, can be seen in Table 1.

Overlapping genes

The 220 Ensembl genes were uploaded to two other databases:

1. UniProt and 2. DisGeNet (<https://www.disgenet.org/home/>), a platform that contains one of the largest publicly available collections of genes and variants associated to human diseases, in order to identify the top 10 overlapping genes. The identification of the overlapping genes between Ensembl and UniProt was established via the STRING database (<https://string-db.org/>). The STRING database collects and integrates information from several sources such as reference publications and experimental data and creates predicted and known protein-protein interactions for a variety of organisms [6]. After uploading the 220 genes in STRING, it matched several of them to UniProt keywords and in this particular case to ‘Amyotrophic lateral sclerosis’. For the documentation of the overlapping genes between Ensembl and DisGeNet, the DisGeNet database was searched for ‘Amyotrophic lateral sclerosis’ related genes. After identifying the top 10 overlapping genes, they were uploaded in the STRING database and a co-expression analysis was performed.

Functional enrichment analysis

Cytoscape (<https://cytoscape.org>) is an open source bioinformatics software platform utilized for the visualization of gene interaction networks [7]. Supplementary features/plugin are available through the Cytoscape app. Through the Cytoscape application, the BiNGO (Biological Network Gene Ontology) plugin was downloaded using the App Manager. Bingo is a Java-based tool that is utilized in order to assess overrepresentation or underrepresentation of Gene Ontology (GO) categories. Using the GeneMANIA interactions dataset, the BiNGO settings were arranged for GO Biological Process, Molecular Function and Cellular Component visualization in Homo sapiens. The size of the nodes represents the number of genes which are annotated to that node. The color scale for GO Biological Process, Cellular Component and Molecular Process for the network visualization is shown in Figure 1. The p-value <0.05 was set as the threshold to indicate statistical significance. The color of the node symbolizes the corrected p-value. White nodes are not significantly over-represented. The color scale ranges from yellow (p-value = significant level) to dark orange (p-value = higher significance level).

Results

Gene network

The gene network of ALS is comprised of 220 nodes and 3342 edges (interactions) as can be seen in Figure 2 and it was downloaded from GeneMANIA. The type of associations (edges) between the genes (nodes) can be seen in Table 1.

Among the 220 genes that Ensembl identified as genes linked with ALS, the top 10 genes from said list that overlapped between three databases (Ensembl, DisGeNet and UniPort) are: 1. SOD1, 2. TARDBP, 3. C9orf72, 4. OPTN, 5. SQSTM1, 6. CHMP2B, 7. VCP, 8. ANG, 9. UBQLN2 and 10. VAPB.

SOD1 in ALS: Mutant superoxide dismutase 1 (SOD1) is one of the most frequently associated genes with ALS along with TARDBP and FUS/TLS [8,9]. It is estimated that approximately 100 single point mutations of copper, zinc SOD 1 are associated with ALS and in particular with familial form of ALS (fALS) [10].

TARDBP in ALS: TARDBP encodes the transactive response DNA binding protein-43 (TDP-43). TDP-43 is known to play a significant role in ALS. As it will be established below, the Gene Ontology (GO) term ‘Cytoskeletal protein binding’ appears enriched in the ALS-related analysis. Studies show that phosphorylated and ubiquitinated TDP-43 result in pathological protein aggregation, inclusion bodies formation and abnormal exosome secretion [11].

C9orf72 in ALS: An established genetic test in order to diagnose ALS is a multigene panel that includes C9orf72, TARDBP and SOD1 [12]. C9orf72 repeats have been shown to be a common cause of ALS. So far, evidence suggests that there are three disease mechanisms associated with C9orf72: 1. Loss of C9orf72 protein function, 2.

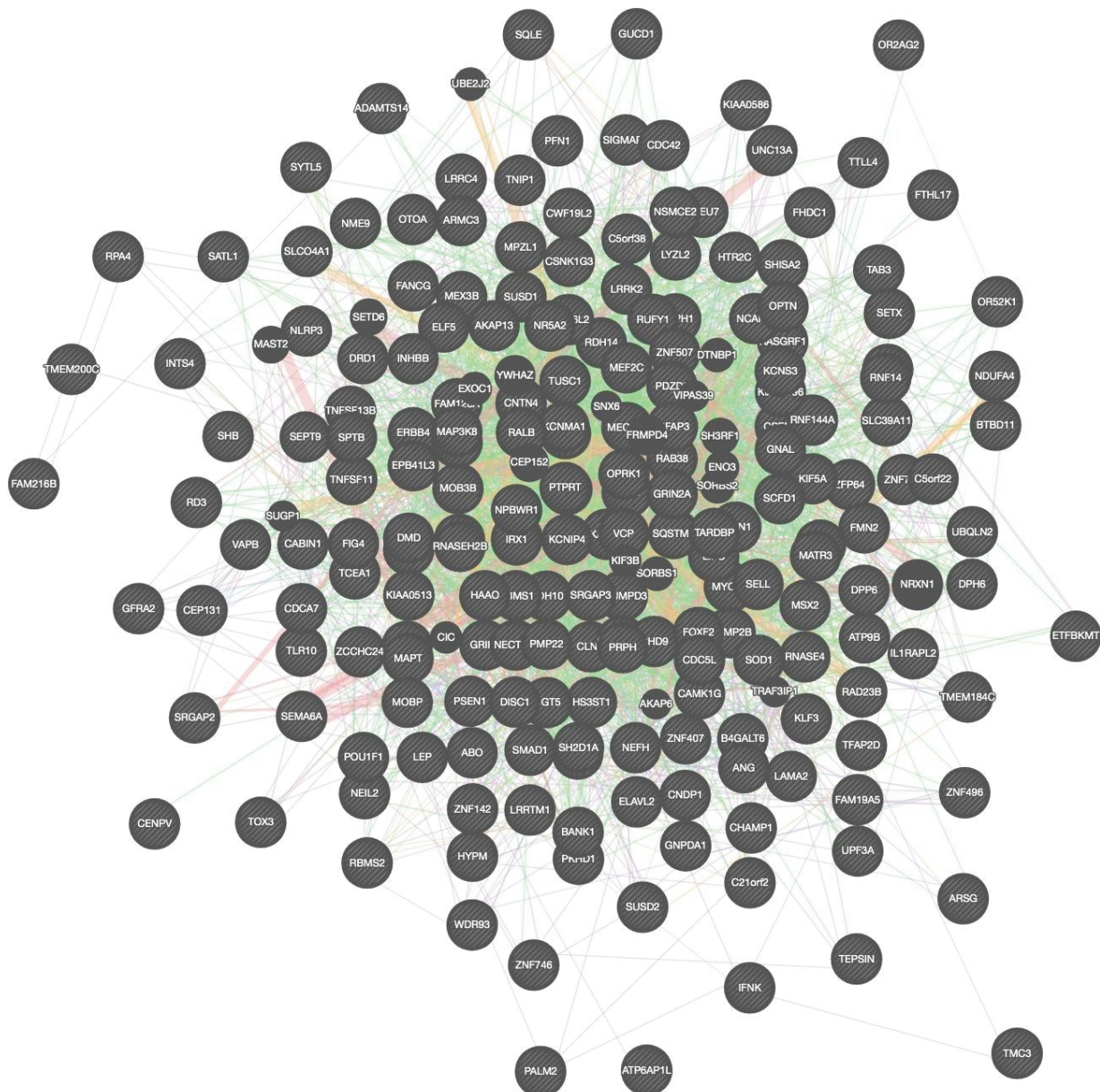


Figure 2: Gene interaction network with 220 nodes and 3342 edges

Toxic gain from repeat expansions in the gene and 3. Production of dipeptide repeat proteins encoded by repeat-associated non-ATG translation [13].

OPTN in ALS: Mutations in this gene have been previously linked with ALS. The OPTN gene encodes optineurin. Optineurin is involved in a variety of cellular processes (e.g. autophagy, neuroinflammation). Researchers proposed that neuroinflammation plays a significant role in the pathogenesis of ALS [14].

SQSTM1 in ALS. p62 is a protein involved in the pathogenesis of neurodegenerative diseases and it is encoded by the SQSTM 1 (Sequestosome-1) gene [15]. SQSTM1/p62 is an autophagy receptor usually found in protein aggregates linked with neurodegeneration [16].

CHMP2B in ALS: The CHMP2B gene encodes the charged multivesicular protein 2B. Evidence suggest that ALS is linked with fronto-temporal dementia (FTD) [17]. Mutations in this gene have been associated with FTD. Reports also show that CHMP2B mutations are present in ALS patients [18].

VCP in ALS: Mutations in the VCP have been associated with

familial type ALS [19]. In addition, exosome sequencing also identified VCP-related mutations in ALS cases [20]. The VCP genes encodes the valosin-containing protein.

ANG in ALS: Mutations in the coding region of angiogenin (ANG) have been identified in ALS patients. More specifically, mutations in ANG, an angiogenic ribonuclease, have been linked with functional loss of ANG activity [21]. ANG is expressed in neurons and it is proposed to have both neurotrophic and neuroprotective roles [22].

UBQLN2 in ALS: Ubiquilin-2 (UBQLN2) plays a significant role in the degradation of overexpressed and misfolded proteins. Gene mutations in UBQLN2 are indicated to play a role in ALS pathology. A possible mechanism of action as proposed by scientists is that UBQLN2 is implicated in the cytoplasmic mislocalization of TDP-43 into insoluble aggregates. UBQLN2 appears to also be involved in several functions such as neuroinflammation and autophagy [23].

VAPB in ALS: Vesicle-associated membrane protein-associated protein B (VAPB) is encoded by the VAPB gene. VAPB mutations form inclusions in the endoplasmic reticulum (ER) [24]. Another study showed that TDP-43, may target VAPB-PTPIP51 interactions

Category	Term	Corrected p-value	Gene Count
BP	Regulation of biological process	2.68E-02	102
BP	Multicellular organismal process	2.49E-02	78
BP	Developmental process	2.67E-02	61
BP	Multicellular organismal development	2.68E-02	57
BP	Positive regulation of biological process	9.29E-03	50
BP	System development	2.67E-02	49
BP	Positive regulation of cellular process	2.63E-02	43
BP	Cellular developmental process	1.31E-02	40
BP	Cell differentiation	1.46E-02	39
BP	Nervous system development	1.02E-02	31
CC	Cytoskeleton	5.41E-04	34
CC	Cell projection	1.15E-06	30
CC	Neuron projection	1.84E-10	26
CC	Cytoskeletal part	5.41E-04	24
CC	Cell projection part	4.70E-06	19
CC	Axon	6.86E-07	15
MF	Protein Binding	3.23E-02	126
MF	Cytoskeletal protein binding	1.67E-02	17

Table 2: Top enriched GO terms of the 220 genes associated with ALS

Gene	Degree	Gene	Closeness Centrality	Gene	Betweenness Centrality
GRIN2A	84	KCNIP4	0.5951087	KCNIP4	0.02984465
PTPRT	81	PTPRT	0.59189189	PTPRT	0.0279715
KCNIP4	79	MECOM	0.57480315	KALRN	0.02582376
PMP22	78	PMP22	0.57329843	PMP22	0.02390078
KIFAP3	74	GRIN2A	0.56589147	GRIN2A	0.01833969
KALRN	73	KALRN	0.56443299	MECOM	0.01755889
RBMS1	69	RBMS1	0.56298201	GNAL	0.01674903
MECOM	66	PDZD2	0.5601023	ATXN1	0.01517189
KCNMA1	63	RDH14	0.55867347	DCTN1	0.01430742
PDZD2	62	KIFAP3	0.55867347	PDZD2	0.01396106

Table 3: Top 10 genes based on degree, closeness and between ness

for damage. VAPB-PTPIP51 interactions regulate ER- mitochondria associations [25].

Functional enrichment analysis

GO biological process: Further analysis of the network was performed through examination of the network's genes for associations with gene ontology annotations. GO Biological Process, Cellular Component and Molecular Function analysis for the 220 genes was performed using the BiNGO Cytoscape plug-in. The genes were mapped to 150 different biological processes and were linked by 245 associations (Figure 3). In terms of node size, the gene expression analysis showed that ALS is strongly associated with: Regulation of biological process (102 genes), Multicellular organismal process (78 genes), Developmental process (61 genes), Multicellular organismal development (57 genes), Positive regulation of biological process (50 genes), System development (49 genes), Positive regulation of cellular process (43 genes), Cellular developmental process (40 genes), Cell differentiation (39 genes) and nervous system development (31 genes). In terms of colour scale, as can be seen from

Figures 1 and 3 there are several functions in yellow that appear to play a significant role. The significance level increases as the colour scale approaches red. The functions with p-values <0.05 include but are not limited to 'death', 'cell death', 'synaptic transmission', 'positive regulation of biological process', 'transmission of nerve impulse', 'nervous system development', 'regulation of neuron projection development', 'neurogenesis', 'generation of neurons', 'neuron development', 'regulation of neuron differentiation' and 'regulation of neuronal synaptic plasticity' Cell death in ALS. Evidence suggests that TDP-43 depletion may be a potential mechanism of neuronal cell death in ALS. Toxic intracellular TDP-43 aggregates could result in neuronal apoptosis [26]. Mutations in SOD1 have also been linked with neuronal programmed cell death [27].

GO cellular component: GO Cellular Component Network yielded 64 nodes and 100 interactions. In this network, the bigger nodes do not appear to have a significant role. However, there are several smaller nodes with high significance levels. As can be seen in the colour scale (Figure 1, section 2.4) and in Figure 4, regarding GO Cellular component, genes were mainly enriched in 'cytoskeleton' (34 genes), 'cell projection' (30 genes), 'neuron projection' (26 genes), 'cytoskeletal part' (24 genes), 'cell projection part' (19 genes) and 'axon' (15 genes). Nodes that appear significant based on the colour scale but are quite small regarding size are 'site of polarized growth' (8 genes) and 'growth cone' (8 genes) (Figure 5).

GO molecular function: For GO molecular function, the network included 13 nodes and 14 edges. In this case, the genes were mostly enriched in 'protein binding' (126 genes) and 'cytoskeletal protein binding' (17 genes). 'Opioid receptor activity' also appears enriched in the colour scale but it is too small in size (only 3 genes are annotated to that node). GO functional analysis demonstrated that genes associated with ALS are mainly involved in 'Cytoskeletal protein binding', 'Protein Binding', 'Multicellular organismal process', 'Positive regulation of cellular process', 'System development', 'Developmental process', 'Regulation of biological quality', 'Multicellular organismal development', and 'Regulation of biological process' (Figure 6 and Table 2).

Cytoskeletal protein binding in ALS: Motor neurons depend on the cytoskeleton for stability, signalling and axonal transport. The Transactivating Response Region (TAR) DNA Binding Protein (TDP-43) forms cytoplasmic aggregates in the motor neurons of patients suffering from ALS [28]. TDP-43 is known to interact with several cytoskeletal components, for example it directly binds to the RNA of the neurofilament light chain. The formation of TDP-43 aggregates results in weakened axon formation and degraded axon length. The report produced by GeneMANIA shows that 3.89% (out of 37.54%) of physical interactions shown in Figure 2, were established by the following article "Global Analysis of TDP-43 Interacting Proteins Reveals Strong Association with RNA Splicing and Translation [29].

Heat map: Genes that appear in the top 10 gene list for degree, closeness and betweenness are shown in Table 3. Genes that overlap in all three categories (degree, closeness and betweenness) include: GRIN2A, PTPRT, KCNIP4, PMP22, KALRN, MECOM and PDZD2. Of the seven aforementioned genes, the following four (GRIN2A, PTPRT, KCNIP4 and PMP22) have been previously linked with ALS by several studies. The relationship of GRIN2A, PTPRT and KCNIP4 with ALS is examined in the next paragraphs. Regarding Peripheral myelin protein 22 (PMP22), PMP22 deletion has been linked with ALS by a variety of evidence [30-33].

The aforementioned genes (top 10 genes based on degree, closeness and between ness) were uploaded to STRING database where a co-expression analysis in Homo Sapiens was performed. The analysis yielded the heat map that can be seen in Figure 7.

Co-expression between genes can predict function association. From the heat-map above (Figure 7), co-expression in the query organism (H. Sapiens) can be observed between **DCTN3** and **DCTN1** (co-expression score of 0.503 based on protein coregulation), **DCTN1** and **DCTN2** (co-expression score of 0.218 based on protein co-

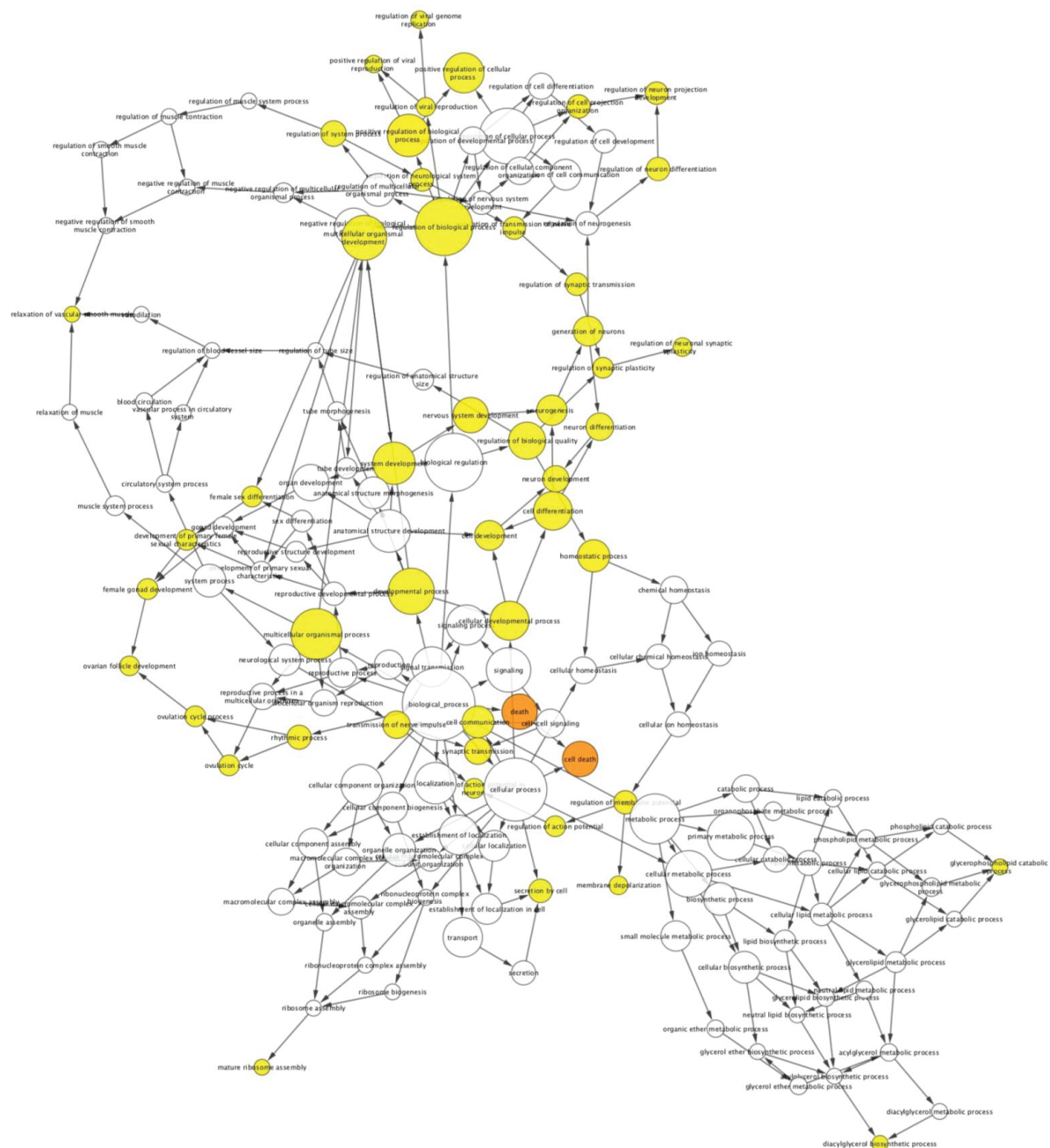


Figure 3: GO biological process network visualization via bingo plug-in in cytoscape

regulation), **PTPRT** and **GRIN2A** (RNA co-expression score of 0.213), **DCTN3** and **DCTN2** (co-expression score of 0.237 based on protein co-regulation) and **PTPRT** and **KCNIP4** (RNA co-expression score of 0.124).

DCTN3 and DCTN1, DCTN1 and DCTN2 & DCTN3 and DCTN2 in ALS: DCTN encodes dynactin (DCTN), a motor protein with a complex structure that comprises of 6 subunits. DCTN1 is the large subunit of dynactin and DCTN3 is the small subunit. DCTN plays a role in the retrograde axonal transport disturbances which have been proposed to be a potential mechanism of ALS pathogenesis [34]. Dynein/dynactin complex is involved in a variety of significant process in the human body. Such functions include axon maintenance, removal of damaged organelles and toxic, misfolded, aggregated proteins from axons to the cell body [35]. Dynein is a cytoskeletal motor protein that generates movement on microtubules [36]. Another study showed that DCTN1, DCTN2, DCTN3, and DCTN4 are co-expressed. DCTN5 showed correlation only with DCTN1 and DCTN6 was co-

expressed with DCTN1, DCTN3, and DCTN4 [37]. Reduced protein levels of DCTN1, reduced DCTN1 mRNA and DCTN1 mutations have been also linked with sporadic cases of ALS [38].

PTPRT in ALS: Several studies have shown that PTPRT is significantly associated with sporadic ALS [39]. The protein tyrosine phosphatase receptor type- T (called PTPR) encodes a protein that belongs to a family of receptor protein tyrosine phosphatases (RPTPs) [40]. This group of proteins is known to play a role in homophilic cell adhesion.

GRIN2A in ALS: There is conflicting evidence of the role of GRIN2A in ALS. A study showed that GRIN2A was significantly up-regulated in the frontal cortex of ALS patients [41]. However, several other studies suggest that GRIN2A is down regulated in patients with sporadic ALS [42,43].

KCNIP4 in ALS: KCNIP4 (Kv channel-interacting protein 4 gene) has been previously linked with ALS [44]. A study showed that KCNIP4

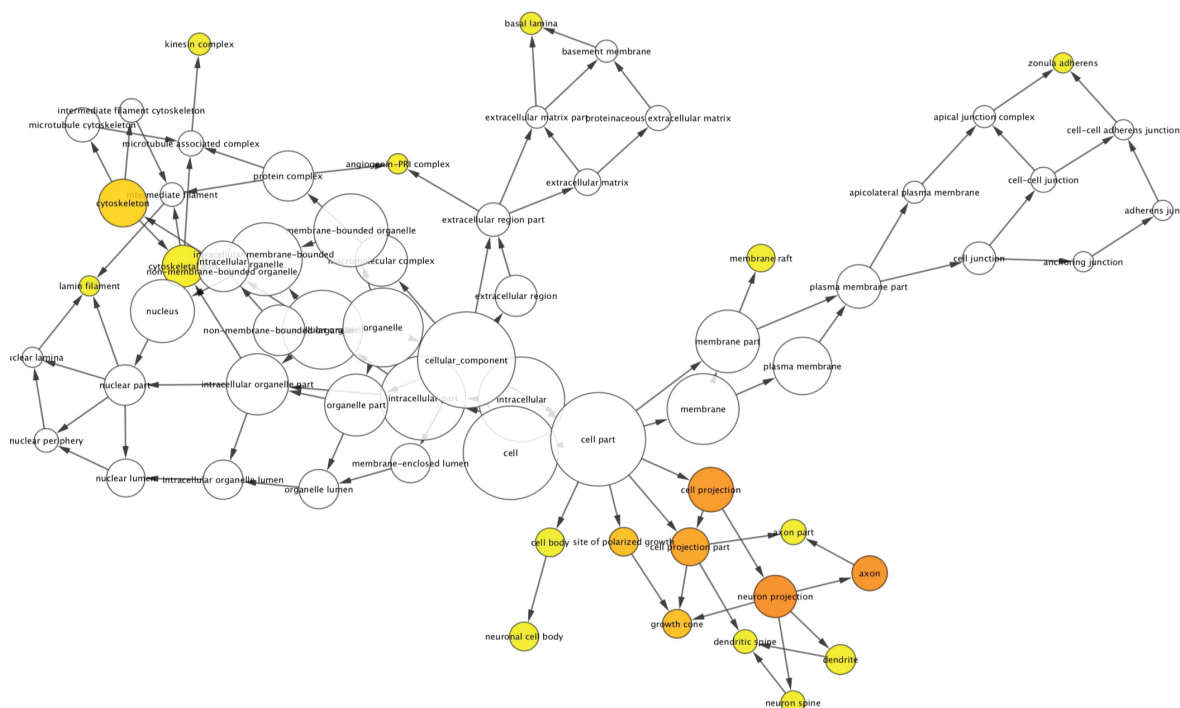


Figure 4: GO cellular component network visualization via bingo plug-in in cytoscape

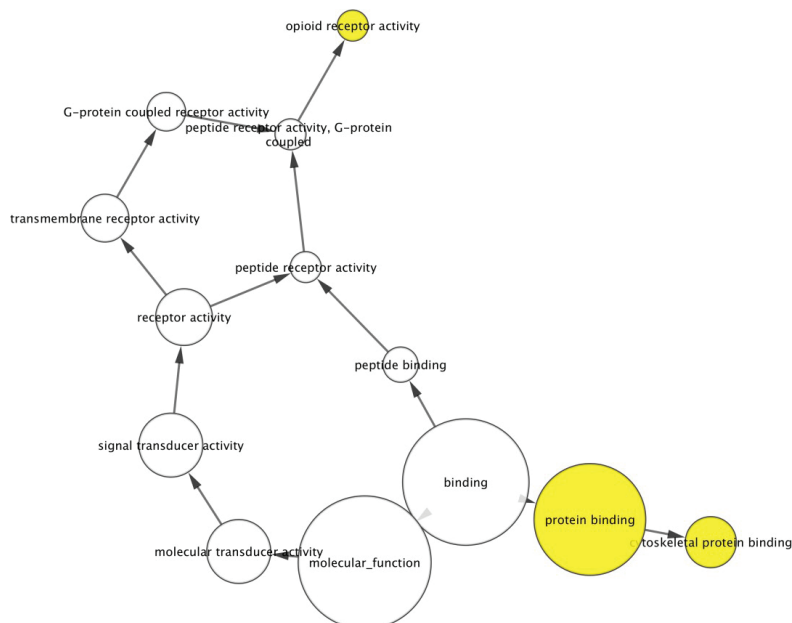


Figure 5: GO molecular function network visualization via bingo plug-in in cytoscape

is down regulated in sporadic ALS motor neurons harbouring TDP-43 cytoplasmic aggregates [45].

Discussion

This study yielded a variety of gene sets as a result of several examination approaches. In regard to overlapping genes between three databases (Ensembl, DisGeNet and UniPort), the following top 10 genes were identified: 1. SOD1, 2. TARDBP, 3. C9orf72, 4. OPTN, 5. SQSTM1, 6. CHMP2B, 7. VCP, 8. ANG, 9. UBQLN2 and 10. VAPB. The role of the aforementioned genes in ALS is supported by a significant amount of evidence from other studies. Studies show a strong association between OPTN and UBQLN2, ALS and

neuroinflammation as well as cell death. Concerning the functional analysis in terms of GO biological process, GO cellular component and GO molecular function the most enriched GO terms are listed in section 3.2 and can be clearly seen in Figure 6 and Table 2. The GO terms with the higher gene count include: 1. Protein Binding (126), 2. Regulation of biological process (102), 3. Multicellular organismal process (78) and Developmental process (61). The GO terms with the lowest p values are 'neuron projection', 'axon', 'cell death', 'death', 'cell projection', 'cell projection part', 'cytoskeleton', 'cytoskeletal part' and 'positive regulation of biological process'. However, a conclusion can't be formed based solely on node size and colour. As can be seen in Figure 4, the part of the pathway that begins with the GO term 'cell

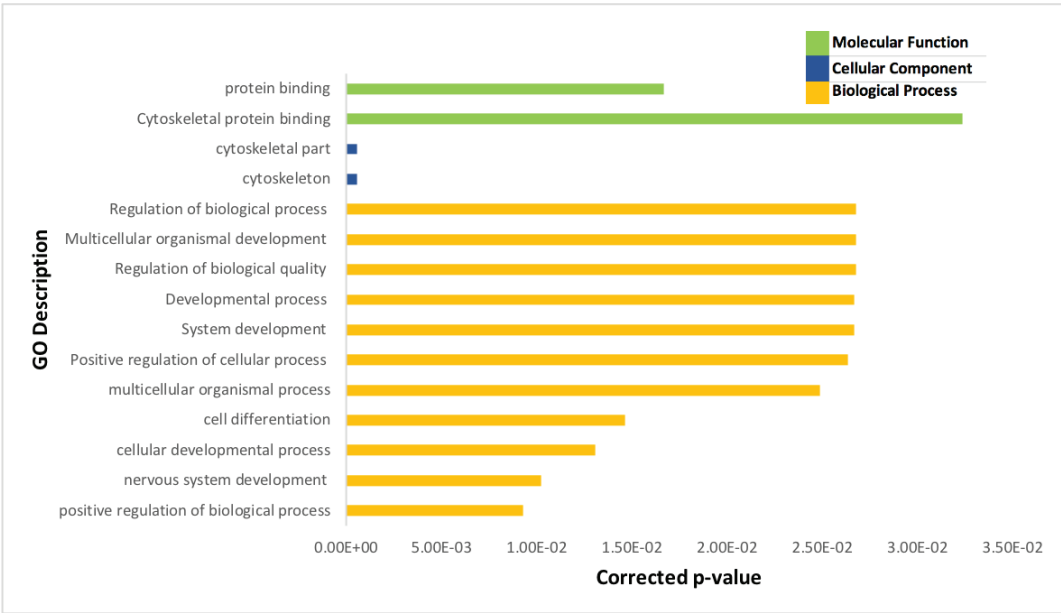


Figure 6: The top 15 enriched GO terms of the 220 genes associated with ALS. The GO terms ‘cell death’, ‘death’, ‘cell projection’, ‘neuron projection’ and ‘axon’ are not included in figure 6 seeing as they have a very low adjusted p value that could not be visible in the chart

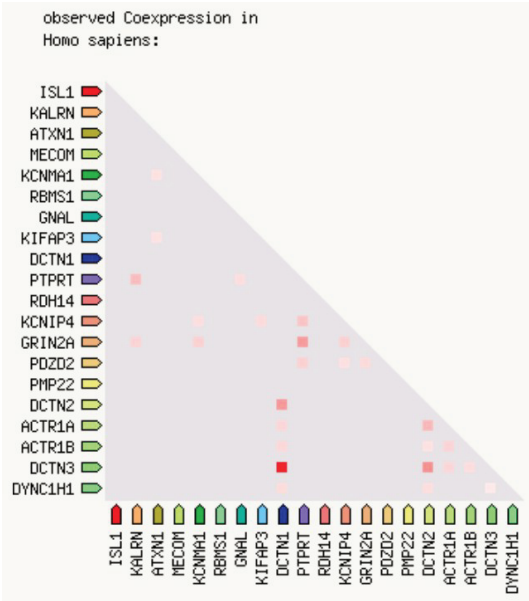


Figure 7: Heat map of 10 genes associated with ALS. In the triangle-matrices above, the intensity of the colour indicates the level of confidence that two proteins are functionally associated, given the overall expression data in the organism. The colour scheme is as follows: (white= low co-expression score to red=high co-expression score).

projection’ (high significance levels) results in the ‘axon part’ and the ‘growth cone’ terms either following the ‘cell projection part’ or the ‘neuron projection’ cascade. This leads to the conclusion that said GO terms could potentially play a role in the disease. Taking in consideration the significance level (colour scale) and the pathway as can be seen in Figure 4, it is evident that the GO term ‘axon’ is a factor worth further studying. In a neuronal cell, important cellular materials are transported along its axon. Any mutation that could impair axon transportation could be examined as a potential mechanism in ALS pathogenesis [46]. In order to identify key genes that show high co-expression levels, a list of genes was established based on their ‘degree’, ‘closeness’ and ‘betweenness’ (as can be seen in Table 3). Subsequently, the genes that overlapped in all three

categories yielded some interested co- expression relationships as can be seen in Figure 7. Examining the published research on those genes, it was established that DCTN1, DCTN2, DCTN3, PTPRT, GRIN2A and KCNIP4 had been previously linked with ALS.

Conclusions

In conclusion, a variety of key genes and pathways are proposed to play a role in the pathogenesis of ALS. However, further research and validation of these genes and their potential mechanisms of action is required. Future work will include identification of differentially expressed genes (DEGs) and hub genes as well as further investigation into the role of axons in ALS pathophysiology.

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