RESEARCH ARTICLE

Protein Network Analysis to Prioritize Key Genes and Pathway for Stress-Mediated Neurodegeneration

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Abstract:

Background:

Oxidative Stress (OS) has been implicated in the pathophysiology of many neurodegenerative diseases. OS can cause cellular damage that results in cell death due to overproduction of reactive oxygen species (ROS) that may play the crucial role in the disease progression. An impaired mechanism in correlation with reduced expression of antioxidant proteins is the very common feature among most of the age-related disorders. Various *in-vitro* and *in-vivo* studies suggest the major contribution of oxidative stress in neurodegeneration. Role of Nrf2 gene is well established as a neuroprotective gene especially in concern with stress-mediated neurodegeneration. Nrf2 is a bZIP transcription factor that forms the heterodimer with small Maf protein and transcription factor AP1 that regulates transcription by binding to ARE which coordinates the transcription of genes involved in phase II detoxification and an antioxidant defense that is used to protect the cell from oxidative stress.

Aim:

The current *insilico* study was attempted to prioritize key genes and pathway in stress-mediated neurodegeneration through network-based analysis.

Methods:

Protein-protein interaction network was constructed and analyzed using 63 Nrf2 regulating candidate genes obtained from NCBI database based on literature studies using *STRING 10.0* database and *Cytoscape v 3.6.0* software plug-in *Network Analyzer.* Further, the functional enrichment analysis of identified gene was done using *PANTHER GENE ONTOLOGY* software and DAVID tool.

Results:

Based on network topological parameter, TP53, JUN, MYC, NFE2L2, AKT1, PIK3CA & UBC were identified as the key gene in the network. Among them, TP53 gene was obtained as a super hub gene with the highest Betweenness Centrality (BC) and node degree. The functional enrichment analysis was done using *PANTHER GENE ONTOLOGY* software and DAVID tool reveals their significant role in neurotrophin signaling pathway, MAPK signaling pathway, cellular response to stress & in the regulation of stress.

Conclusion:

The network analysis will help in prioritizing genes in the pathway that helps in understanding the underlying mechanism of disease. Thus, further study on these genes and their biological mechanism and pathway may, therefore, provide a potential target for the treatment of stress-mediated neurodegeneration.

Keywords: Neurodegenerative diseases, Oxidative stress, Nrf2, Protein-protein interaction network, STRING 10.0, Cytoscape 3.6.0.

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1. INTRODUCTION

Neurodegenerative diseases are the multifactorial disorder, which results in slow progressive loss of neurons [1, 2]. The etiology of neurodegenerative diseases is still not clear; however, it results in diverse factors such as oxidative stress, Endoplasmic Reticulum (ER) stress, mitochondrial dysfunction, accumulation of Reactive Oxygen Species (ROS), loss of mitochondrial membrane potential, and ATP depletion [3 - 5]. The neurological disorders, such as Alzheimer's [6], Parkinson's [7], Huntington's [8], Amyotrophic lateral sclerosis [9], Multiple Sclerosis [10], and other processes related to pathological aging [11] are associated with accumulation of abnormal protein aggregates in and around affected neurons. Oxidative stress and protein misfolding play a key role in the pathogenesis and progression of neurodegenerative diseases [12] that are characterized by fibrillar aggregates composed of misfolded proteins [13]. Neuronal death or apoptosis can be mediated by either oxidative stress or ER stress or by both at the cellular level. Thus, there have been continuing efforts to find out a target that can protect the cell against oxidative damage and have the potential to treat neurodegenerative diseases.

Decrease levels of antioxidants result in contemporizing of free radicals. In the recent years, the area of research interest focuses on the endogenous cellular anti-oxidative responses *via* signaling pathways involving Nrf2 [14]. Nrf2 is a bZIP belongs to the cap'n'collar family, responsible for activating transcription mediated by Antioxidant Response Element (ARE) in response to oxidative stress [15]. ARE is a cis-acting enhancer found in the 5′ flanking region of many phase II detoxification and cytoprotective genes. The ARE protects against oxidative stress, mitochondrial dysfunction, and misfolded protein. Nrf2 activity is dependent on Kelch-like ECH-associated protein 1 (Keap 1) [16], is a repressor protein that binds with Nrf2 that leads ubiquitination and promotes degradation by the proteasome [17]. On oxidation of sulfhydryl groups on specific Cysteine or Phosphorylation of Keap1, the interaction between Nrf2 and Keap is disrupted. Stabilized Nrf2 translocates into the nucleus, where it's bind to small Maf protein and modulates transcription through ARE. The formed heterodimers bind to the ARE and coordinate the transcription of genes involved in phase II detoxification and antioxidant defense [18]. Proteins expressed by these phase II detoxifying and antioxidant genes are used to maintain redox balance as well as protect the cell from oxidative stress.

Activation of the Nrf2 pathway can boost the ability to buffer free radical generation as well as it also provides new therapeutic intervention for the treatment of neurodegenerative diseases. Nrf2 has proven activity in animal models of many neurological disorders supporting the concept of developing drug target to activate the Nrf2 pathway in the brain.

In the AD, oxidative stress has the major influence on Amyloid Precursor Protein (APP) processing and tau modification *via* a sequence of critical events that leads to increased brain toxicity which results in oxidative stress [19]. The cell culture studies admit the toxic effect of Aβ42 on the brain that leads to cell death *via* apoptosis [20]. Neurotoxic compounds, such as N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine or its active derivative, MPPT and 6-hydroxydopamine (6-OHDA) exposure lead to oxidative stress, impair mitochondrial respiration and energy metabolism that results in neurodegeneration [21]. In the AD, blocked Nrf2 activity may lead to the neuronal dysfunction and/or loss [22]. The study on substantia nigra region of human PD brain revealed confirmation of protein glycation and nitration that leads to the oxidative damage to DNA and protein resulting from persistent oxidative trauma [23]. Comparative gene expression study of neurosphere taken from PD olfactory mucosa between PD patient and control reveals significant imbalance Nrf2-ARE pathway in PD patient [24]. In ALS, Nrf2 activators play important role in protection against oxidative stress and cell death induced by SOD1 mutant protein [24, 25]. Astrocytes expressions of Nrf2 increase the lifespan and enhance the motor neuron survival in the spinal cord of SODG93A and SODH46R/H48Q transgenic mouse model of ALS [26]. Thus increase in transcriptional activity of Nrf2, will decrease the risk and delay the aging [27, 28].

On the other hand, the activity of Nrf2 is also regulated by phosphorylation through several kinases such as phosphoinositol-3 kinase (PI3K), extracellular signal-regulated protein kinase (ERK), Protein Kinase C (PKC), and pancreas enriched kinase (PERK) [29 - 37] that are localized in mitochondria, a major source of intracellular oxidative stress [38]. The direct phosphorylation of Nrf2 by PERK disrupts the interaction between Nrf2 and Keap1 that leads to nuclear translocation [39]. PERK kinases activated in response to Endoplasmic Reticulum (ER) stress. It has been suggested that cell survival after ER stress is mediated by the increase in Nrf2-induced glutathione [40]. A major cause of ER stress is the deposition of unfolded proteins [41], due to two of the major pathological features of the AD (amyloid plaques and NFTs) are composed of misfolded proteins. ER stress is activated in neurons and astrocytes at some point during AD progression. Upregulation of ER stress markers has been demonstrated in postmortem brain tissues and cell-culture models of many neurodegenerative disorders, including PD, AD, Amyotrophic Lateral Sclerosis

(ALS), and Huntington disease and Spinocerebellar ataxias [42]. In the AD, β-amyloid aggregation induces ER stress, altering ER and mitochondrial morphology and increasing ER and oxidative stress [43]. In PD, accumulation of misfolded proteins leads to the up-regulation of UPR. In the study, ATF6a, an ER-membrane-bound transcription factor, has been shown to be activated by protein misfolding in the ER in order to protect dopaminergic neurons from MPTP [44]. Thus, Nrf2 has proven activity in animal models of many neurological disorders supporting the concept of developing a drug target to activate the Nrf2 pathway in the brain.

Computational methods have been used widely for the identification of potential drug targets in different disease pathogenesis. Genomics-based approach combined with system biology is an efficient way to identify the set of functionally enriched genes from among the arrays of the potential gene in the network. The current *insilico* study is performed to identify key genes and pathway against stress-mediated neurodegeneration and their functional enrichment analysis based on protein-protein interaction network approaches.

2. METHODOLOGY

2.1. Data Collection

We mined sixty-three Nrf2 regulating gene candidates in human from NCBI database on the basis of literary studies. (Table **1**) (www.ncbi.nlh.nic.gov).

Table 1. List of the sixty-three Nrf2 regulating genes in human from literature and database mining.

Gene Symbol	Gene ID	Gene Description
TP53	7157	tumor protein p53
BRCA1	672	DNA repair associated
AKT1	207	AKT serine/threonine kinase 1
PPARG	5468	Peroxisome proliferator-activated receptor gamma
PTEN	5728	Phosphatase and tensin homolog
CDH1	999	Cadherin 1
MYC	4609	v-myc avian myelocytomatosis viral oncogene
CDKN1A	1026	Cyclin-dependent kinase inhibitor 1A
CDH1	999	cadherin 1
PIK3CA	5290	Phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic alpha
HMOX1	3162	heme oxygenase 1
RB1	614041	RB transcriptional corepressor 1
NFE2L2	4780	Nuclear factor, erythroid 2 like 2
GSK3B	2932	Glycogen synthase kinase 3 beta
JUN	3725	Jun proto-oncogene, AP-1 transcription factor
AHR	196	aryl hydrocarbon receptor
PRKCA	5578	Protein kinase C alpha
HDAC1	3065	Histone deacetylase 1
UBC	7316	Ubiquitin C
CREBBP	1387	CREB binding protein
PRKCD	5580	Protein kinase C delta
NQO1	1728	NAD(P)H Quinone dehydrogenase 1
FBXW11	23291	F-box and WD repeat domain containing 11
SQSTM1	8878	Sequestosome 1
SUMO1	7341	Small ubiquitin-like modifier 1
HDAC2	3066	Histone deacetylase 2
SMARCA4	6597	SWI/SNF related, matrix associated, a regulator of chromatin
MAP2K1	5604	Mitogen-activated protein kinase 1
HDAC3	8841	Histone deacetylase 3
YY1	7528	YY1 transcription factor
BTRC	8945	Beta-transducin repeat containing E3 ubiquitin protein ligase
CASP3	836	Caspase 3
KEAP1	9817	kelch-like ECH associated protein 1

(Table 3) contd.....

Gene Symbol	Gene ID	Gene Description
CLTC	1213	Clathrin heavy chain
ATF4	468	Activating transcription factors 4
EIF2AK3	9451	eukaryotic translation initiation factor 2 alpha kinase 3
MAFK	7975	MAF bZIP transcription factor K
MAPK9	10155	Tripartite motif containing 28
TRIM28	5598	Mitogen-activated protein kinase 7
GADD45GIP1	90480	GADD45G interacting protein 1
CUL3	6613	Small ubiquitin-like modifier 2
SUMO2	5605	Mitogen-activated protein kinase 2
MAP2K2	5583	Protein kinase C eta
PRKCH	7832	BTG anti-proliferation factor 2
BTG2	6478	Siah E3 ubiquitin protein ligase 2
SIAH2	571	BTB domain and CNC homolog 1
BACH1	6047	Ring finger protein 4
RNF4	6942	Transcription factor 20
COPS7A	50813	COP9 signalosome subunit 7A
CASP1 ENC1	834 8507	Caspase 1 ectodermal-neural cortex 1

2.2. Construction of PPI Network

We made use of *STRING 10.0* (Search Tool for the Retrieval of Interacting Genes), for obtaining direct and indirect human protein-protein interaction network [42]. The STRING database provides functional associations derived from sources including database, experimental, co-expression, text mining, co-occurrence, neighborhood *etc* with the highest confidence score. We constructed network based on the highest confidence score of 0.04, which implies that only interactions with high level of confidence in network considered as reliable PPI network. Then, the PPI constructed by STRING 10.0 was visualized by *Cytoscape v 3.6.0,* software used for biological network visualization, data integration and interactive network generation [43].

2.3. Topology Analysis of PPI Network

The protein-protein interaction (PPI) network was analyzed using Cytoscape Plug-in *Network Analyzer 3.6.1* based on parameter including betweenness centrality (BC) and node degree. In the network, gene represents node and edges represent the interaction between nodes. The degree indicates no. of edges linked to nodes, the highest the degree of nodes represents significant biological function [44]. Betweenness centrality defines the importance of the node based on the number of shortest paths that pass through each node. In the study, the PPI network was analyzed based on these parameters.

2.4. Functional Enrichment Analysis

Functional enrichment analysis of nodes in cluster network was performed using *PANTHER GENE ONTOLOGY Tool* [45] and *DAVID* (The Database for Annotation, Visualization, and Integrated Discovery Functional Annotation Tool) [46] for understanding the biological relevance of genes in response to ER/oxidative stress. GO ontology provides common detail framework to functionally annotated gene sets. KEGG provides information about molecular interaction and network reaction as the pathway. PANTHER gene list analysis used statistical overrepresentation test to analyze functionally enriched gene network in GO Biological process. The Statistical overrepresentation test uses binomial statistical comparison. DAVID Functional Annotation tools analyze functionally enriched gene in KEGG Pathway. The functional enrichment analysis was performed based on Bonferroni correction, fold enrichment and P-values as a statistical parameter (Fig. **1**).
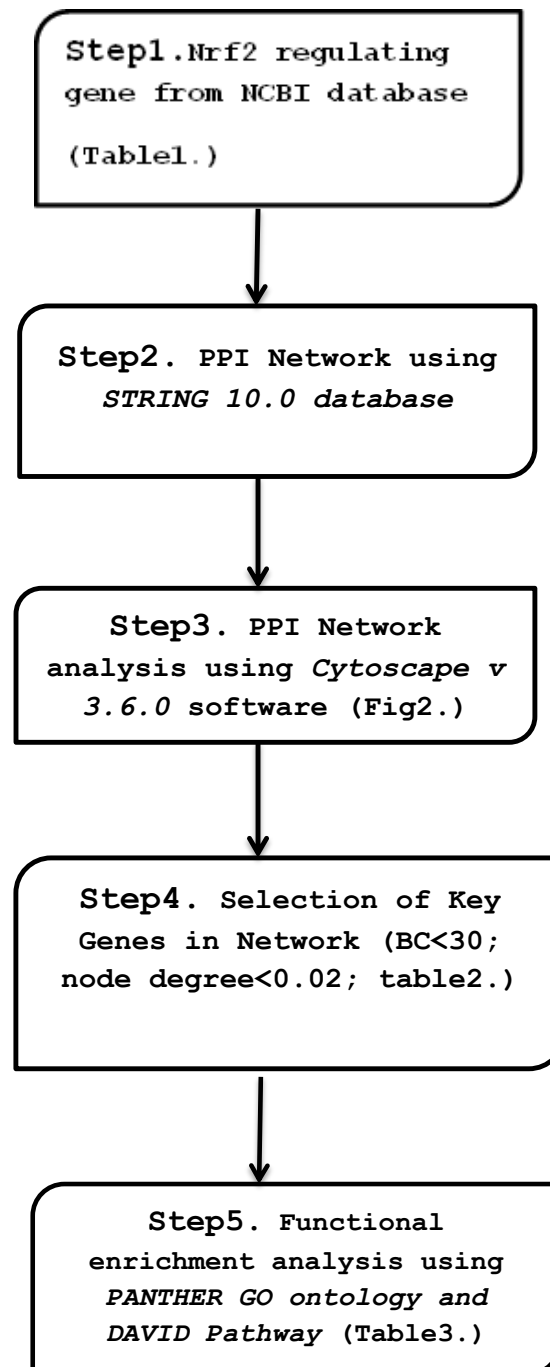
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Step1. Nrf2 regulating
gene from NCBI database

(Table1.)
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Step2. PPI Network using
      STRING 10.0 database
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Step3. PPI Network
analysis using Cytoscape v
3.6.0 software (Fig2.)
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Step4. Selection of Key
Genes in Network (BC<30;
node degree<0.02; table2.)
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Step5. Functional
enrichment analysis using
PANTHER GO ontology and
DAVID Pathway (Table3.)
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Fig. (1). An overview of methodology.

3. RESULTS AND DISCUSSION

Network-based approach is used to construct network [47] from large gene data set that leads to the prediction of putative candidate genes, prioritizing drug targets from the network [48]. The study focuses on the inter-relationship between the various components using the PPI network and assists in the identification of novel genes associated with the disease. Studies have employed a PPI network-based approach to identify the important novel gene in response to stress-mediated neurodegeneration [49, 50]. We have taken into consideration Nrf2 regulating genes implicated in response to ER and oxidative stress-mediated neurodegeneration in human.

3.1. PPI Network

Network analysis provides information about the molecular and cellular interactions of genes/protein within the

network [51]. It represents entities (nodes) and their functional co-relationships (edges). Each data type contains a different aspect of the functional role of interested genes. In the study, we investigate protein-protein interaction using available data and knowledge-based using *STRING 10.0* database. PPI network constructed using 63 genes in *STRING* as input that results in 547 interactions between 63 nodes based on parameters including database, experimental, co-expression, text mining with the confidence score (0.007), average degree nodes 17.4 and average local clustering coefficient 0.621.
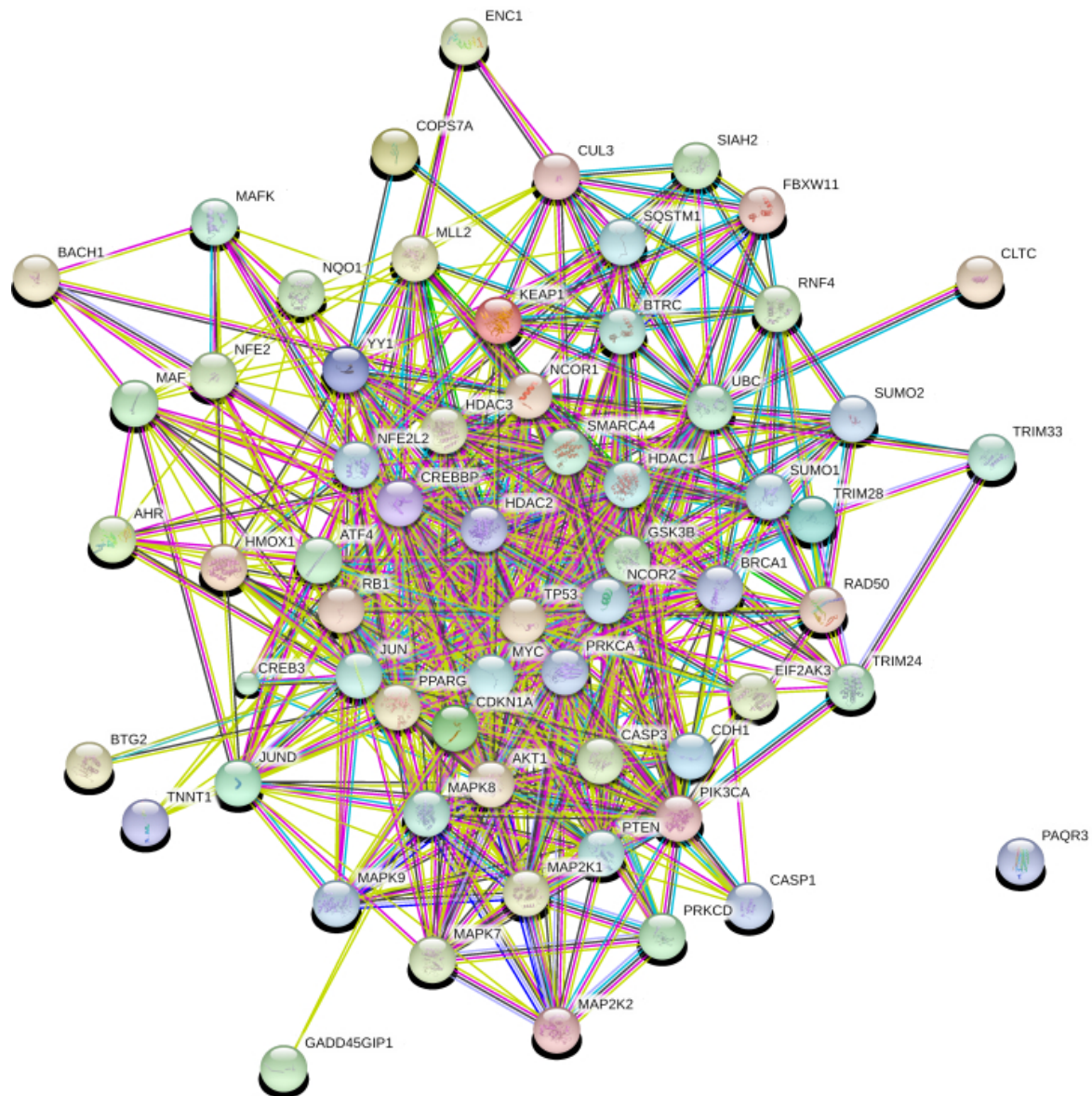
Fig. (2). Overview of PPI Network constructed using *STRING 10.0* database. The network includes 547 edges (interaction) between 63 nodes respectively based on experiment, co-expression, text mining & co-expression with 0.007 confidence score as the analysis parameter.

3.2. PPI Network Analysis

The network obtained from *STRING* was subsequently analyzed and visualized using Cytoscape 3.6.0 plugin Network Analyzer. In the study nodes with high degree and BC value taken as the key parameter to analyze the network Fig. (**2**). A cut off value for BC > 0.02 and node degree >30 consider as topological parameter for gene prioritization. TP53 (tumor protein p53), JUN (Jun proto-oncogene), UBC (Ubiquitin C), NFE2L2 (nuclear factor, erythroid 2 like 2), PIK3CA (Phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic alpha), AKT1 (AKT serine/threonine kinase 1) and

MYC (v-myc avian myelocytomatosis viral oncogene) are a hub genes in network based on cut off value Table **2**. Among these, TP53 is super hub gene having the highest betweenness centrality and node degree in the network (Fig. **3**).

Table 2. Obtained Key Genes in Network based on Topological parameter based on the cutoff value of BC < 0.02 & Node degree <30. Among them, TP53 is obtained as a super hub gene in the network with the highest BC value & Node degree.

Gene	Node Degree	Betweeness Centrality (BC)
TP53	50	0.13322847
JUN	43	0.06460235
MYC	39	0.0442512
NFE2L2	35	005194822
AKT1	34	0.02486275
PIK3CA	32	0.02158976
UBC	31	0.09984772
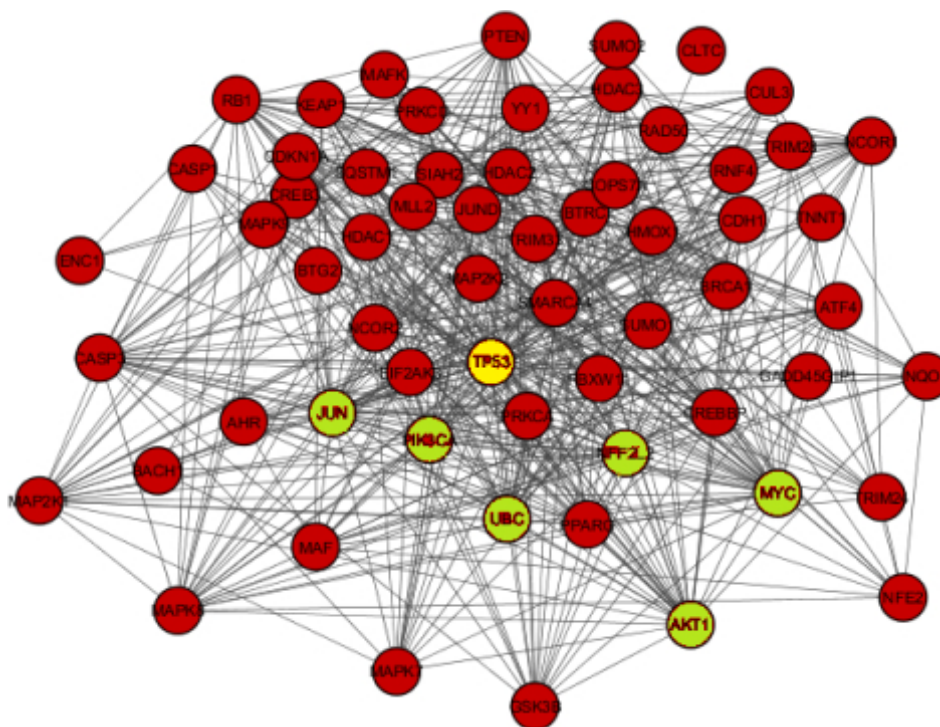
Fig. (3). Overview of PPI Network constructed using Cytoscape_3.6.0. Network includes 547 edges (interaction) between 63 nodes respectively. The node with yellow and green colors represents the key genes in the network with cut off value BC > 0.02 and node degree >30. Among key genes, the node with yellow color represents the superhub gene with highest betweenness centrality and node degree.

3.3. Function Annotation Analysis

For the better understanding of functional annotation of key genes in the network, we run enrichment analysis by using *PANTHER GENE ONTOLOGY* software and DAVID. In PANTHER Gene ontology, GO biological process reveals enrich genes including Regulation of response to stress, cellular response to stress Table **3**. Pathway analysis mainly includes KEGG pathway reveals that genes are commonly enriched in neurotrophin signaling pathway, MAPK signaling pathway (Table **3**).

Table 3. The result of Functional Enrichment analysis of Key genes in PPI Network.

ID/Pathway	Term	P-value	Associated Genes
Go_0033554	Cellular response to stress	1.89E-06	[TP53, UBC, AKT1, MYC, NFE2L2]
Go_0006950	Response to stress	4.79E-03	[TP53, UBC, AKT1, MYC, NFE2L2, PIK3CA]
Go_0080134	Regulation of response to stress	2.85E-03	[JUN, TP53, NFE2L2 UBC, AKT1, MYC, PIK 3CA]
KEGG04722	Neurotrophin signaling pathway	1.51E-10	[JUN, TP53, AKT1, PIK3CA]
KEGG04010	MAPK signaling Pathway	2.6E-5	[JUN, TP53, AKT1, MYC]

4. DISCUSSION & CONCLUSION

Oxidative stress and protein misfolding initiate apoptotic cascades and are known to play significant roles in the pathogenesis and progression of neurodegenerative disease. Thus, targeting the Nrf2 pathway is becoming the most promising neurodegenerative therapy as it is known to induce expression of the variety of cytoprotective and detoxifying genes. Serval studies reveal the potential role of Nrf2 regulating the gene in protection from stress-induced neurodegeneration. Systems biology approaches generously contributing towards the extensive data generation which can be fruitful for better understanding as well as better cures for the stress-induced neurodegeneration.

The main aim of the study is to identify key genes and pathway against stress-mediated neurodegeneration using protein-protein interaction network analysis. The constructed PPI network consisted of total 547 interactions between 63 nodes. Based on network topology parameter *i.e.* Betweenness Centrality (BC >0.04) and node degree (> 30) TP53, JUN, MYC, NFE2L2, AKT1, PIK3CA & UBC were identified as the key gene in the network. Among which TP53 gene was obtained as a super hub gene with highest Betweenness Centrality (BC) and node degree. Their enrichment analysis reveals their active role in MAPK signaling pathway & neurotrophin signaling pathway.

The p53 tumor suppressor (TP53) gene regulates cell survival and death as well as cellular-redox homeostasis *via* modified expression of pro and anti-oxidant protein that affect mitochondrial Reactive Oxygen Species (ROS) production [52]. In the network, TP53 acts as a significant node in the intracellular control pathway, in the monitoring of cellular response to the various level of stress cell cycle arrest, replicative cell senescence, DNA repair, *etc*. Microglial apoptosis and microglial-induced neurotoxicity significantly reduce the treatment of microglia with p53 inhibitor pifithrin-α (PFTα) revealing the neuroprotective role of microglia p53 pathways in Alzheimer disease [53]. JUN gene is the transcription factors (AP-1) that encode the c-JUN protein that regulates pro-inflammatory cytokines, oxidative and other forms of cellular stress, and UV irradiation. The signaling pathway possesses the neural and non-neural cell that can sense oxidative stress and activate adaptive response against stress that provides strength to the anti-oxidant system. The low level of ROS, induce transcription factor nuclear factor erythroid-derived 2-related factor 2 (Nrf2) gene which is responsible for induction of many genes including NADPH Quinone oxidoreductase (NQO1), glutathione S-transferase, Heme Oxygenase-1(HO-1), ferritin, *etc*. [54]. Another hand, an average increase in ROS, activates AP-1 that regulates c-JUN, c-FOS *etc* genes. Hence, the activation of Nrf2 and AP-1 regulating genes plays the vital role in neural cell survival and protection against oxidative stress as Nrf2 form heterodimer with c-Jun that binds to ARE and regulates the transcription of many phase –II detoxifying gene in anti-oxidant defense network in the Nrf2/ARE pathway. Tp53 protein possesses an anti-oxidant property that activates the transcription of many Nrf2 coding genes and also maintains the mitochondria function in concern with ROS production [55]. In response to activation of the p53 gene by ROS, induce the Mitogen-Activated Protein Kinases (MAPKs) pathway such as p38 and c-Jun N-terminal Kinases (JNK) [56]. MAPKs (JNK & p38) pathway follow by heme- oxygenase 1(HO-1) that counteract the effect of ROS [57]. Nrf2 also interacts with and affects the Notch signaling pathway that influences cell differentiation, survival, and apoptosis. Notch signaling pathway targets the gene expression of Nrf2 coding gene and MAPKs signaling pathway *via* the Ras/MAPK pathway that leads to the activation of the defense system and protects against internal/external stress [58]. Thus, neuron activates the genes and induces a pathway to prevent cell death and apoptosis. The polyubiquitin gene *U*BC is upregulated on activation of Nrf2–Keap1 pathway under oxidative stress condition. *UBC* is found near the Nrf2-ARE binding site [59]. *Ubc* is highly demanded gene under various stress condition, thus providing valuable information underlying the mechanism of Ub in cellular defense pathway [60]. Nrf2 also regulate through an alternative mechanism including phosphorylation of Nrf2 by various protein kinases including PI3K/Akt pathway. Thus, these genes play important roles in providing strength to cellular antioxidant defenses and protect tissues from harmful damage by exerting neuroprotective, antitumor, anti-inflammatory, and antiapoptotic effects [61 - 65].

The network-based analysis help in prioritizing key genes network from among the vast array of potential genes in the PPI network. PPI network analysis is a key mechanism to understand all the biological processes from system biology perspective as well as also used in prediction and evaluation of corresponding treatments, providing a theoretical basis for the search of novel drug targets. In the study, we identified TP53, JUN, MYC, NFE2L2, AKT1, PIK3CA & UBC genes that play the significant role in regulating stress-mediated neurodegeneration using network-based analysis. While experimental evidence also confirms the true potential of these gene candidates in animal & human model. Further, the additional study is required to justify these finding and their potential therapeutic role in disease pathway.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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