

Network and Pathway Analyses of Exercise Therapy on Cerebral Infarction Related Genes

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Abstract

Background: Few efforts were made to conduct a comprehensive analysis of potential genes within the action of exercise therapy on cerebral infarction (CI).

Methods: We used RNA sequencing (RNA-seq) and weighted gene co-expression network analysis (WGCNA) to provide more important insights information than the conventional single-gene analyses. We performed RNA-Seq on the macroscopically preserved and lesioned SD rat CI model (N=4 pairs). The WGCNA constructed a correlation network and identified the modules in the dataset by the dynamic tree-cutting algorithm. We defined the module membership and identified modules associated with external traits. Functional annotation was performed with the Cluster Profiler based on Gene Ontology and KEGG database.

Results: We identified 675 associated genes from RNA-seq. WGCNA defined 38 modules, and 12 modules were found with high connectivity with exercise therapy on CI. Cluster Profiler found 250 significant enrichment pathways. Enrichment results indicated some key metabolic pathways, such as the WNT signaling pathway, cAMP signaling pathway, calcium signaling pathway, Hippo signaling pathway, MAPK signaling pathway, cGMP-PKG signaling pathway.

Conclusion: In this study, we found that important biochemical pathways related to exercise therapy on CI. Such a systematic and comprehensive exploration of the genes will help us to identify potential biomarkers for further research.

Keywords: Cerebral infarction, RNA-seq, Weighted gene coexpression network analysis, Functional enrichment analysis

Introduction

Cerebral infarction (CI) is a common cerebrovascular disease with a high disability rate, high recurrence, and high rate of mortality [1,2]. Incidence rates for patients less than 45 years range from 3.4 to 11.3 cases per 100,000 people [3]. The

pathogenesis of CI includes calcium overload in brain tissue cells, brain tissue apoptosis, overexpression of inflammatory factors, free radical injury, acidosis, and the disequilibrium of energy metabolism [4]. Increased research demonstrates exercise therapy has great treatment effects on CI [5-7]. Many genes and pathways are related to the occurrence

and development of the disease [8-10], but the important biomarker associated with exercise therapy was limited known. The underlying mechanism of treatment effects of exercise for CI requires further investigation.

The study has analyzed exosomal microRNAs of cerebrospinal fluid in ischemic stroke rats after physical exercise CI [11], but few efforts were made to conduct a comprehensive analysis of potential genes within a pathway or a network framework. A systematic analysis of pathogenicity genes within pathways or a network framework could provide more important insights than the conventional single-gene analyses.

In this study, we used high-throughput RNA sequencing (RNA-Seq) to explore the exercise effects on genome-wide in the middle cerebral artery occlusion (MCAO) model. Furthermore, we used weighted gene co-expression network analysis (WGCNA) to investigate the biological context and relationships of CI genes identified by the RNA-Seq analyses. The results of this study might provide new insight into the molecular mechanisms of exercise therapy on CI through a systems biological way.

Materials and Methods

Focal cerebral ischemia rat model

MCAO model: The MCAO model was established as reported [12]. Adult male Sprague-Dawley (SD) rats (210±30 g) were obtained from the Chong-qing Bo Aimadisen Biological Technology Co., LTD. The experimental animals were approved by the Experimental Animal Ethics Committee of Southwest Medical University (Approval ID: swmu20220044). Animal experiments were carried out by the institutional guidelines. All rats were housed in individual cages in a controlled temperature environment (25°C±1°C). Rats were kept under a 12-h dark to light cycle, with food and water ad libitum. A third-party researcher used a random number generator (Microsoft Excel, 2007, Bellevue, WA) to select assignment: Group 1 (Sham-exercise group, n=4) without exercise therapy, Group 2 (exercise group, n=4) treated with exercise therapy after the middle.

Exercise training protocol

All rats were trained to run on a treadmill (ZH-PT; ANHUI Zhenghua Biologic Apparatus Facilities, China). The exercise group consisted of a 3-week exercise training duration. The intensity of the exercise was a 2-min warm-up exercise at 5 m/min, then 30 min at 20 m/min, 5 days a week for 3 weeks. Sham-exercise group rats were placed on a stationary treadmill for 30 min each time, with the same treatment schedule. Electrical shocks forced animals to run forward. If rats were unable to run at 20 m/min after global ischemia, a lower speed was used. All rats were sacrificed after the exercise therapy by

using pentobarbital sodium (50 mg/kg intraperitoneally). The focal resulting CI included necrotic and penumbra areas were received after the intervention.

RNA sequencing

Total RNA was extracted using RNAiso Plus (Takara Bio Inc., Shiga, Japan) and a QIAGEN RNeasy® Mini kit (QIAGEN, Hilden, Germany). RNA integrity and quantity were finally measured using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit (NEB, USA). The statistical power of this experimental design, calculated in RNASeqPower is 4. The clustering of the index-coded samples was performed on a cBot-Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). Illumina Novaseq platform sequenced the preparation library with 150 bp paired-end reads after cluster generation.

Weighted gene co-expression network analysis (WGCNA)

A signed weighted correlation network was constructed by creating a matrix of pairwise correlations between all pairs of genes chosen by variance. Correlation coefficients were measured between expression levels of genes. Groups of co-expressed genes (modules) were identified. The connectivity measure (topological overlap) was calculated for each gene by summing the connection strength with other genes. A scale-free signed network was constructed with the optimal soft threshold (power of $\beta=3$). We identified 38 modules in the dataset with a dynamic tree-cutting algorithm and merging threshold function at 0.25. WGCNA was conducted by the statistical analysis software WGCNA R package¹³ [13]. We summarized the expression profile of each module by the corresponding module eigengene. Module eigengene-based connectivity (MEC) defined the module membership for each gene to each module as the Pearson correlation between the expression level and the module eigengene.

Genes with the greatest module membership values are defined as intramodular hub genes. We used Cytoscape Software (3.7.1) to visualize the top 40 gene connections. Intramodular hub genes (genes with the highest MEC values) usually are centrally located inside the module. To identify modules associated with external traits, we calculated the module eigengenes of each module. Pearson correlation coefficients were used to the correlated module with the external traits eigengenes. Modules with p-values <0.05 were identified as trait-related modules.

Functional annotation was performed with the ClusterProfiler based on Gene Ontology and KEGG databases. The top 20 most enriched (ordered by p-value) function terms were visualized. GO term enrichment analysis including molecular function, biological process, and cellular component was used.

Results

Differential gene expression

The normalized gene counts table (Table S1) was subjected to differential expression analysis for all the contrasts. The final numbers of differentially expressed genes were (per contrast): for the contrast exercise group vs control group, 675 statistically significant genes were found with a threshold (p-value = 0.05 logFC=0, 259 were up-regulated, 416 were down-regulated). The Differential Expressed Genes (DEGs) volcano plot (Figure 1), depicts how well samples from different conditions cluster together according to their expression values.

Weighted gene coexpression network analysis

Genes with a low variance of expression signal across samples have little information for inferring interaction, so the most varying 7,020 genes were chosen for network construction.

Constructing a weighted gene network entails the choice of the soft thresholding power $\beta=3$ to which co-expression similarity is raised to calculate adjacency. R function picks a soft threshold that performs the analysis of network topology and chooses a proper soft-thresholding power with 14 (Figure 2a).

The approximate straight line relationship shows approximate scale-free topology (Figure 2b).

Scale-free topology is approximately satisfied when high power is chosen for defining the adjacency matrix. Poor fit to scale-free topology indicated the presence of outliers.

WGCNA was applied to investigate gene sets that were related to traits using the gene expression data of 8 samples. After using a dynamic tree-cutting algorithm, a total of 38 distinct co-expression modules containing grey to turquoise genes per module were identified, and 18 uncorrelated genes

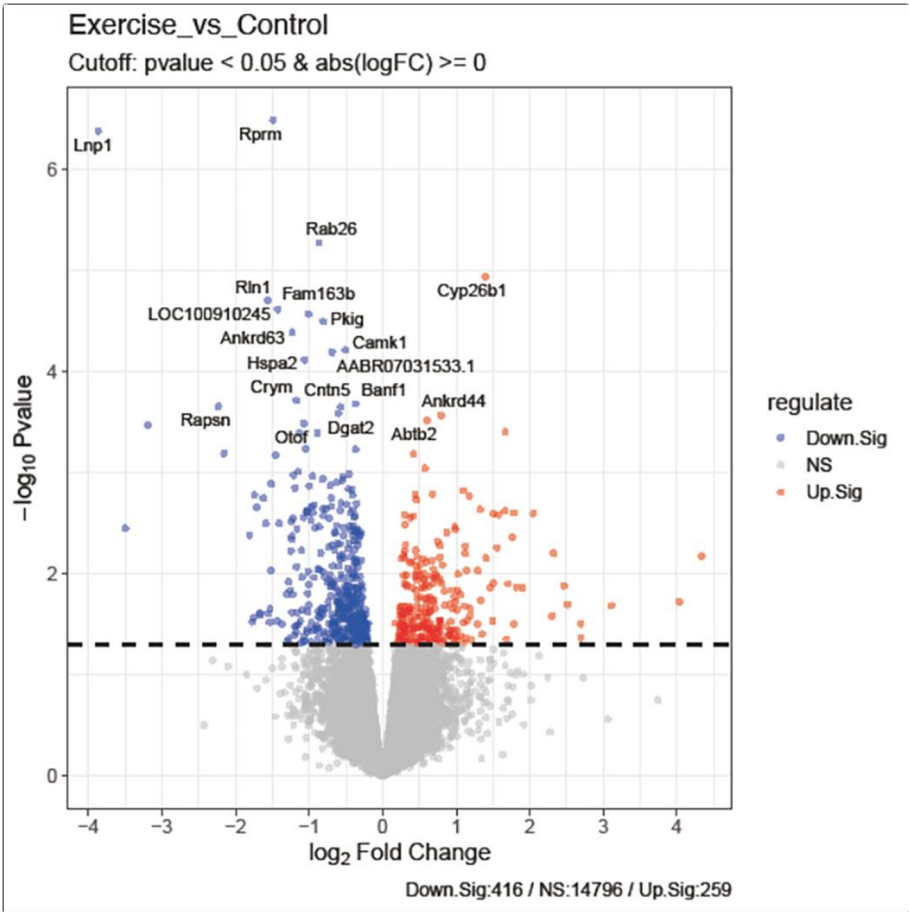


Figure 1. Differential Expressed Genes volcano plot. Combines the results of a statistical test (aka, p-values) with the magnitude of the change enabling quick visual identification of those genes that display large-magnitude changes. The horizontal dashed line sets the threshold for statistical significance, while the vertical dashed lines set the thresholds for biological significance.

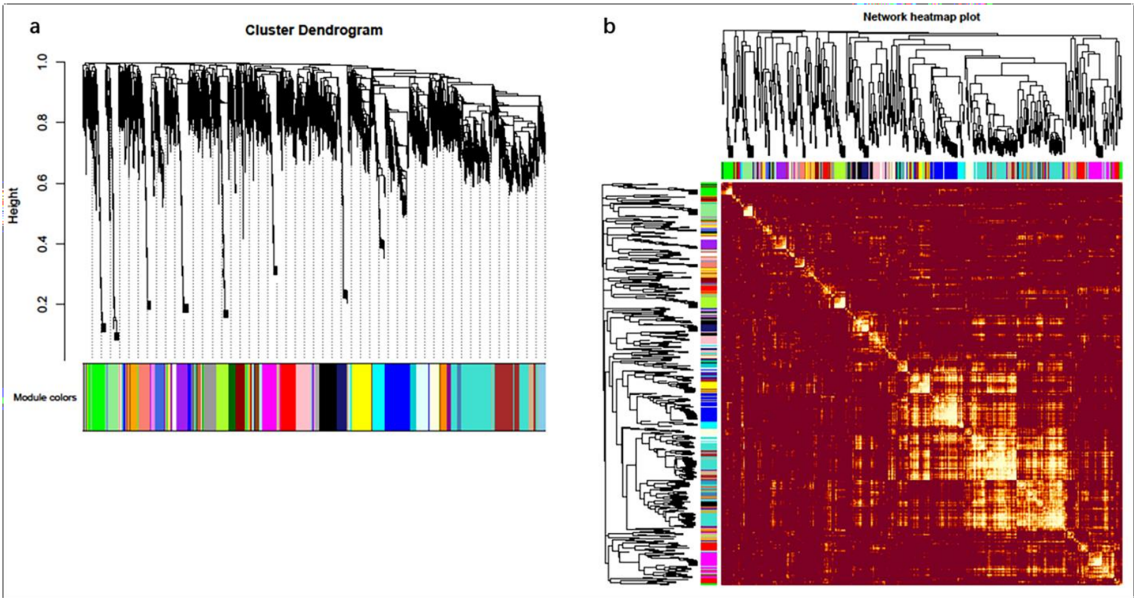


Figure 2. a. Clustering dendrogram of genes and modules identified by weighted gene co-expression network analysis for exercise therapy on cerebral infarction. **b.** The gene network heatmap plot. Light color represents low overlap and the progressively darker red color represents higher overlap. Blocks of darker colors along the diagonal are the modules. The gene dendrogram and module assignment are also shown along the left side and the top.

were assigned into a grey module (**Figure 3a**). A heatmap plot depicting the topological overlap matrix supplemented by hierarchical clustering dendrograms and the module color (**Figure 3b**). The expression of genes in the module can be

summarized into module eigengene (ME). Bar plot is used to visualize the relation between gene expression ME in the module (**Figure 4a**).

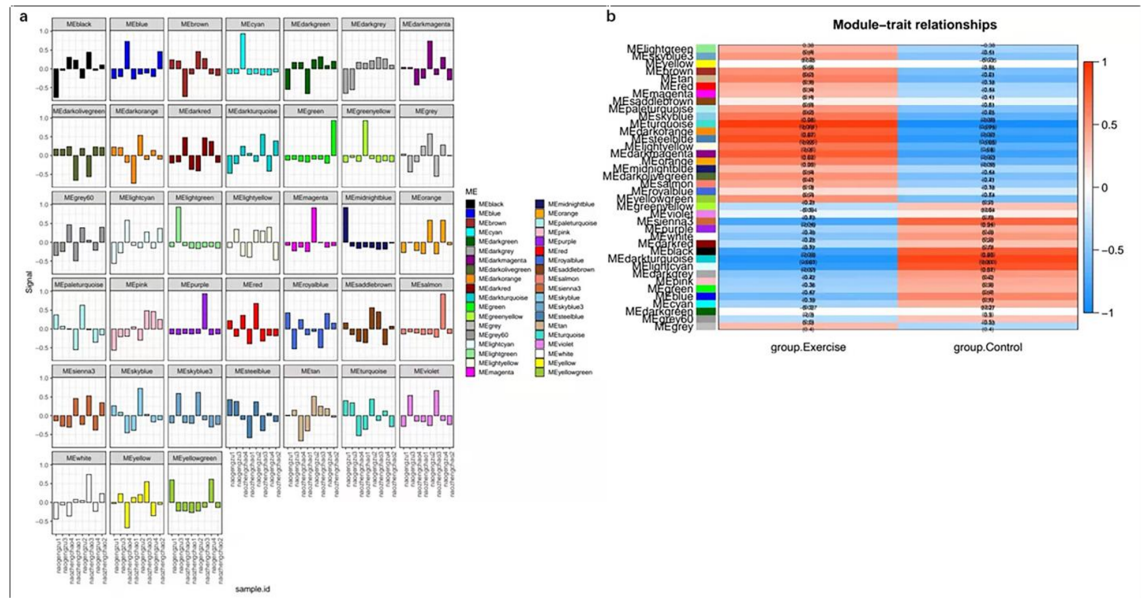


Figure 3. a. Bar plot of ME. The corresponding ME expression values (y-axis) versus the same microarray samples. The module eigengene takes on low values in arrays where a lot of module genes are under-expressed (green color in the heatmap). The ME takes on high values for arrays where a lot of module genes are over-expressed (red in the heatmap). **b.** Heatmap of module-trait correlation. Each row corresponds to a ME, column to a trait. Each cell contains the corresponding correlation and p-value. The table is color-coded by correlation according to the color legend. ME: Module Eigengene.

To understand the biological significance of the modules, we correlated the 38 ME with traits of interest and searched for the most significant associations. According to the heatmap of module-trait correlation, rows indicate modules, and columns indicate external traits (**Figure 4b**). For example, genes clustered in the blue module had the strongest positive correlation with the group.

Module significance can also be defined as the average gene significance of all genes in the module. For a given trait, we can analyze whether average gene significance across modules differs, and different average gene significance means some modules have a strong correlation with the trait (**Figure 5**). For each module, a quantitative measure of module membership

(MM) as the correlation of the module eigengene and the gene expression profile was defined.

Genes with the greatest module membership values (**Table S2**) are referred to as intramodular hub genes and the top 40 hub genes were chosen for each module. Hub genes usually are centrally located inside the network, such as KALRN, KCNAB2, Rac1, LAMP5, RIM-BP2, and CaMKII (**Figure 6a**). To facilitate biological interpretation, we need to know genes in the modules, whether they are significantly enriched in certain functional categories etc [11]. For each module and each function database, the top 20 most significantly enriched term is visualized (**Figure 6b**).

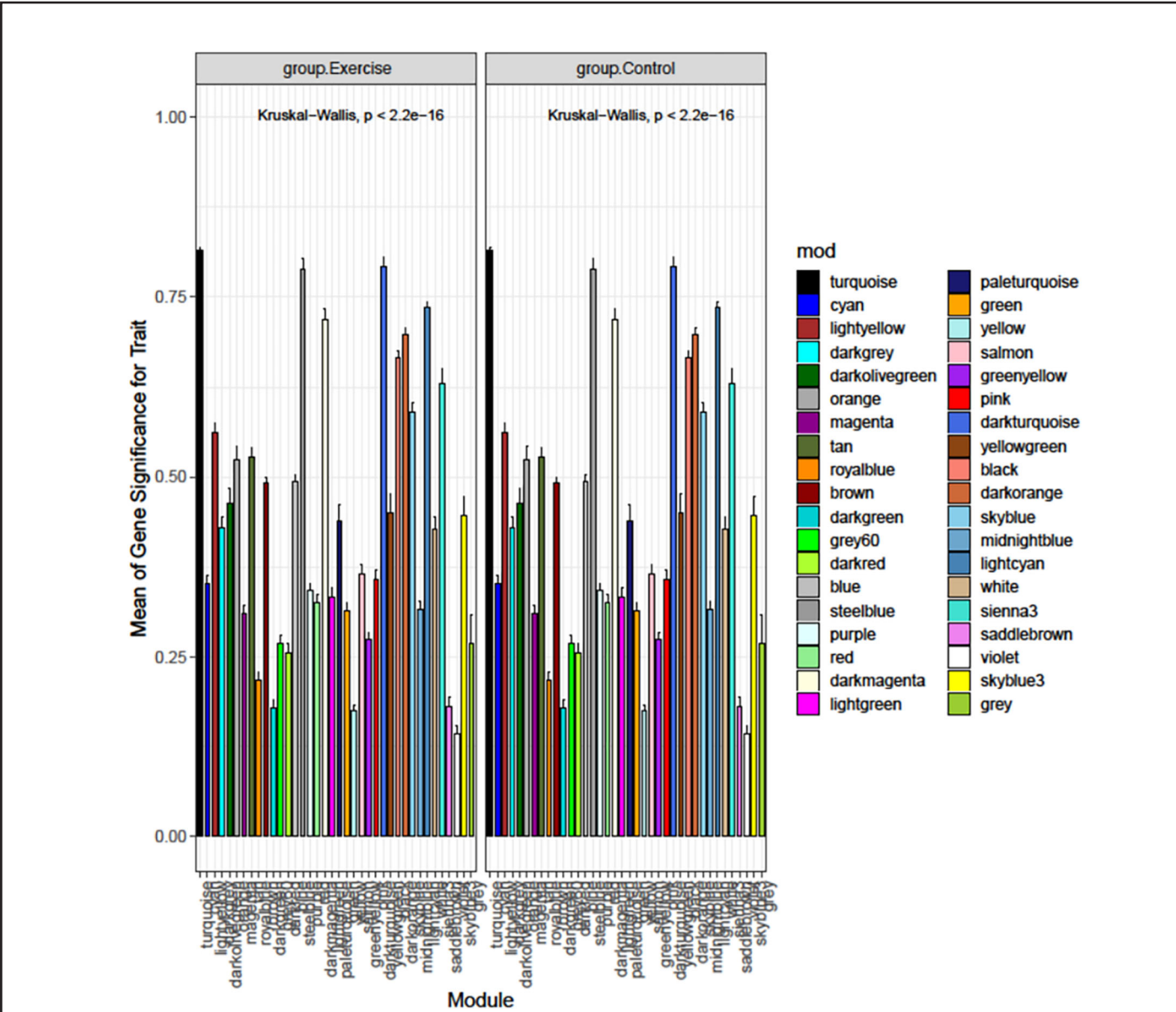


Figure 4. Barplot of module significance defined as the mean gene significance across all genes in the module.

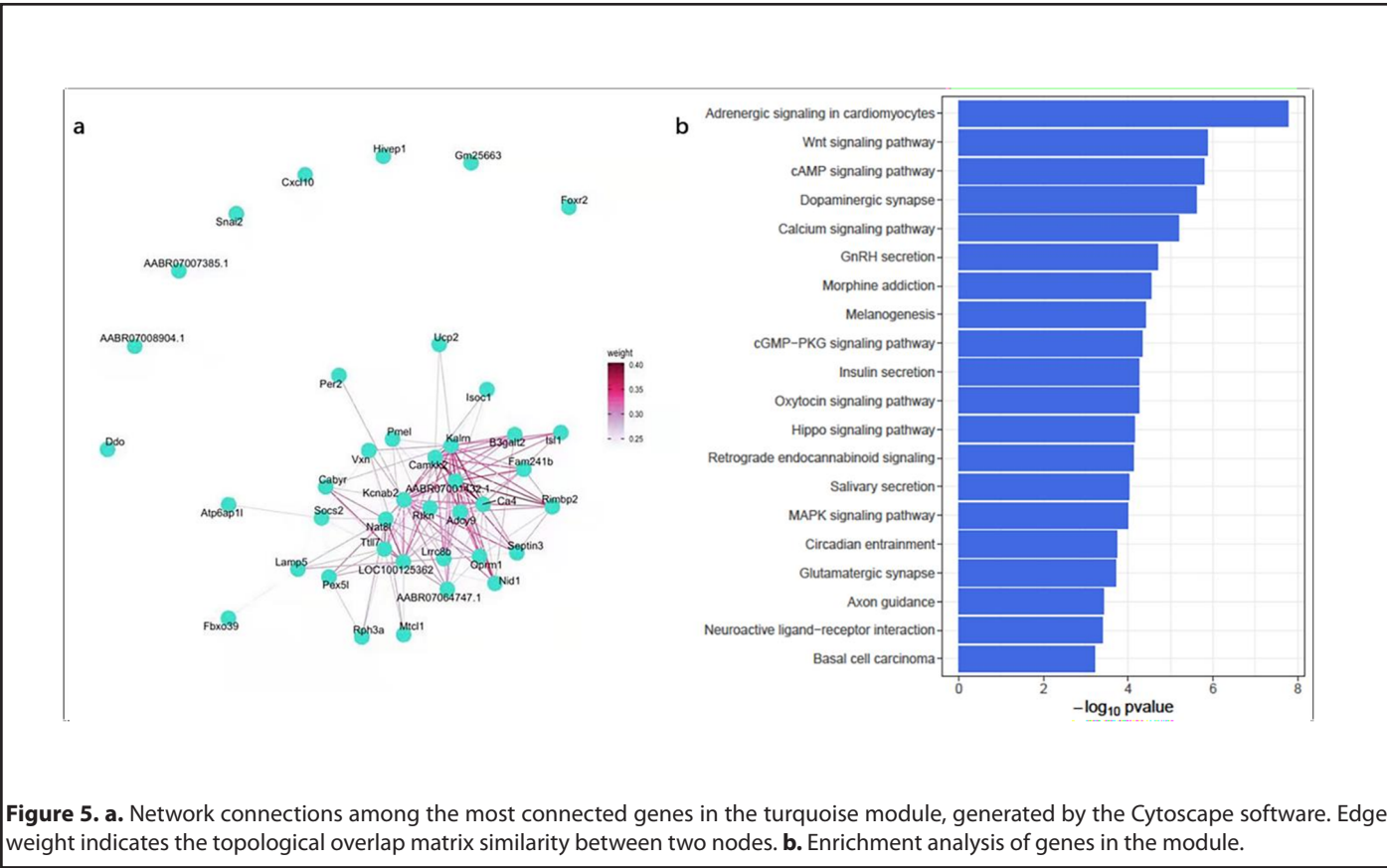


Figure 5. a. Network connections among the most connected genes in the turquoise module, generated by the Cytoscape software. Edge weight indicates the topological overlap matrix similarity between two nodes. **b.** Enrichment analysis of genes in the module.

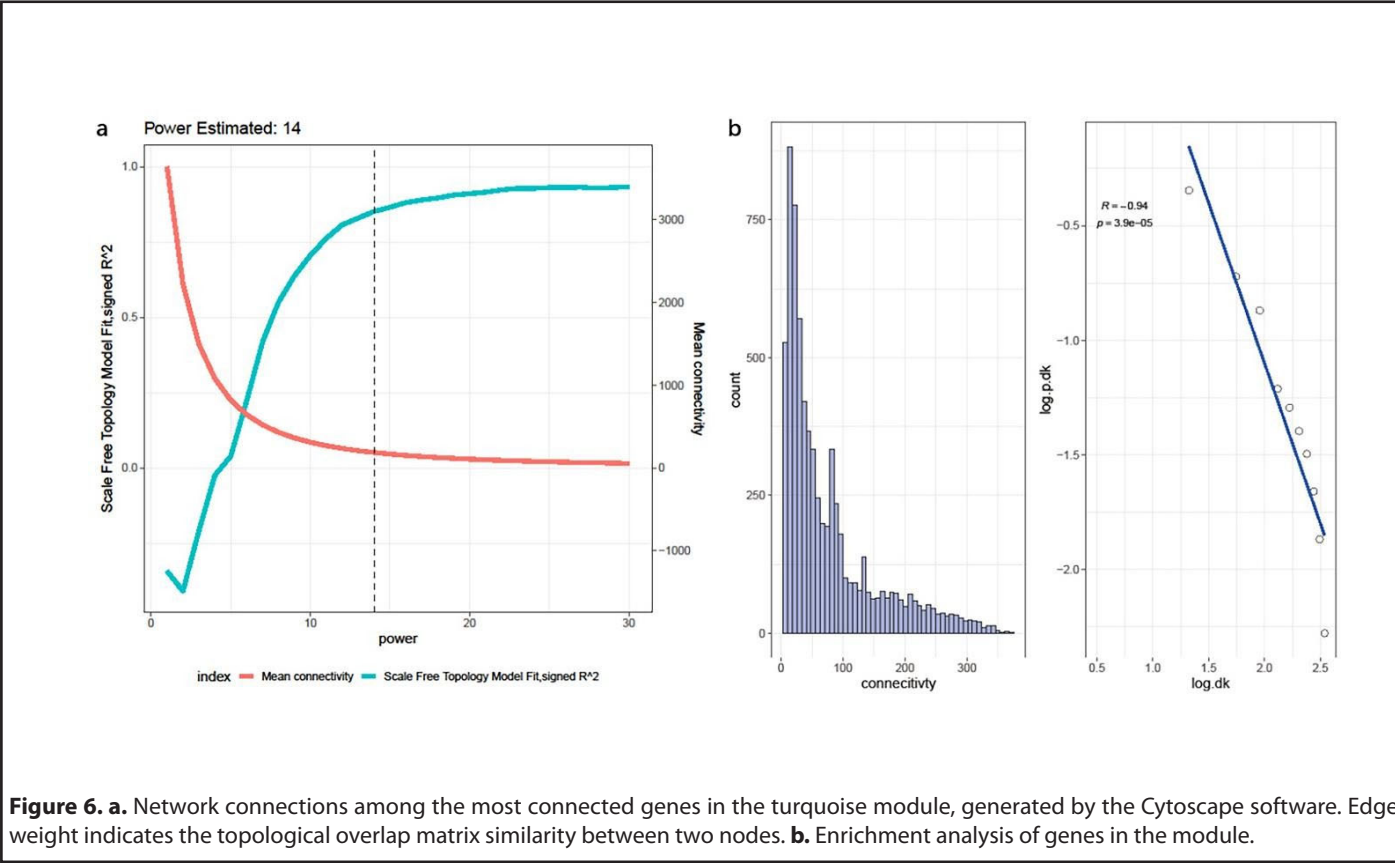


Figure 6. a. Network connections among the most connected genes in the turquoise module, generated by the Cytoscape software. Edge weight indicates the topological overlap matrix similarity between two nodes. **b.** Enrichment analysis of genes in the module.

We used Cluster Profiler to search for abundant pathways in the CI gene set and found 125 significant enrichment pathways for CI. The KEGG significantly enriched in the candidate genes were considered to be the candidate genes, which were relatively reliable for following up bioinformatics analysis. Functional enrichment analysis found the more specific functional patterns. The KEGG terms of substantial enrichment in candidate genes, including those associated with the following pathways, WNT signaling pathway, cAMP signaling pathway, calcium signaling pathway, Hippo signaling pathway, MAPK signaling pathway, cGMP-PKG signaling pathway. These results suggested that the candidate genes collected were reliable for subsequent bioinformatics analysis

Discussion

In this study, we used the RNA-Seq technique to collect transcriptomic profiles from the rat MOCA model after exercise therapy. These results may provide valuable information for exploring the molecular mechanisms underlying. We applied WGCNA on RNA-Seq data to further investigate some important mRNA (KALRN, KCNAB2, Rac1, LAMP5, RIM-BP2, and CaMKII) up-regulated comparison with exercise therapy from CI rats.

KALRN is a novel genetic risk factor for CI [14]. It is involved in the inhibition of inducible nitric oxide synthase, in the regulation of ischemic signal transduction, and in neuronal morphogenesis, plasticity, and stability. KALRN strongly associates with CI in a Chinese Han Population [15]. KALRN could promote smooth muscle cells migration and proliferation to be a guanine nucleotide exchange factor for Rac1 [16]. Deletion of the mouse homolog of KCNAB2 results in associative memory impairments, and amygdala hyperexcitability exercise affects recovery from CI. LAMP5 is a member of the LAMP family of membrane proteins, specifically in the brain. It is involved in controlling the dynamics of evoked GABAergic transmission [17]. GABA acts in a non-synaptic fashion to maintain neural stem, exercise therapy could induce activation of neurogenesis requires a reduction of GABA receptors [18]. Also, LAMP-5 is found to be a novel inflammatory regulator in acute leukemia [19].

All neuro synapses require fusion-competent vesicles and coordinated Ca^{2+} -secretion coupling for neurotransmission [20]. Presynaptic protein RIM-BP2 has important effects on neurotransmitter release [21]. RIM-BP2 has a substantial effect on neurotransmitter release by promoting vesicle docking/priming and vesicular release probability [22].

The ligand Wnt3a promoted the expression of genes encoding presynaptic proteins RIM-BP2 increased [23]. Treadmill exercise has been found to improve neurogenesis and promote neurological function in CI through upregulation of Wnt3a [24]. The important potential effect of RIM-BP2 induced by exercise in CI needs further exploration.

Additionally, Ca^{2+} /calmodulin (CaM)-dependent protein kinase II (CaMKII) is highly abundant in the brain. CaMKII can activate and translocate from the cytoplasm to the postsynaptic density in response to neuronal activation [25]. Treadmill exercise could enhance synaptic plasticity in MCAO mice by increased CaMKII expression [26].

Exercise therapy is primary physiotherapy in CI patients. Lee IM found vigorous exercise associated with decreased stroke risk in men [27]. The voluntary exercise intervention maintained basal dendritic spine density within the focal CI area [28]. The surviving neural circuitry adaptive remodeling may contribute to promoting recovery. Weimin Shen shows that treadmill exercise enhances synaptic plasticity in the MCAO mice [26]. Intensive treadmill training was also found to promote cognitive recovery after CI rats [29]. Although exercise therapy has found recovery effects in CI, the potential mechanism was still unknown. Bioinformatics could provide more valuable information.

Recently, Yitao He has investigated the functions of fluoxetine and the identification of fluoxetine-mediated circRNAs and mRNAs in MCAO mice [30]. There were 879 circRNAs and 815 mRNAs between sham and MCAO groups. However, there is limited intensive bioinformatics analysis for the influencing networks of co-regulated genes. WGCNA is powerful in identifying co-expression modules and we have used it to identify gene expression profiles and key genes in subchondral bone of osteoarthritis [31]. Pathway analyses that take into account the biological relevance of genes provide a data-driven, circumscribed set of high-yield gene targeting exercise therapy for CI.

Biological function enrichment analysis determined a more specific function pattern involved by the CI gene set. As revealed by our KEGG enrichment analysis, these pathway-related-CI participated in Such as the WNT signaling pathway, cAMP signaling pathway, calcium signaling pathway, Hippo signaling pathway, MAPK signaling pathway, cGMP-PKG signaling pathway. We also found that the GO biological process terms of neuron differentiation, nervous system development, calcium iron transport, and canonical Wnt signaling.

The Wnt signaling pathway is a complex regulatory network, which is currently considered to include three branches, Wnt/ β -catenin, planar cell polarity pathway, Wnt/ Ca^{2+} [32]. The Wnt/ β -catenin pathway regulates stem cell differentiation, organ development, and regeneration.

Treadmill exercise has been found to enhance the activation of the Wnt/ β -catenin signaling pathway in the ischemic penumbra in CI through upregulation of Wnt3a and β -catenin protein [24].

The results found another important pathway, the MAPK

pathway, has crosstalk with the Wnt/ β -catenin signaling pathway in brain development and cancer [33,34]. The study found resistance exercise affects catheter-related thrombosis in rats through MAPK/NF- κ B pathway [35]. Rönn identified 2,560 significant transcripts differentially expressed before vs. after exercise, pathways enriched in response to the WNT and MAPK signaling pathways were down-regulated after exercise [36]. Also, the activation of MAPK conducted synaptic plasticity in the lateral amygdala through the cGMP-PKG signaling pathway. Swimming exercise could inhibit myocardial ER stress in the hearts of aged mice by enhancing cGMP-PKG signaling [37]. Dan Lu identified blood circular RNAs for acute ischemia, the same the Hippo signaling pathway was found in our result [38]. Exercise can improve skeletal muscle function in age rats and inhibit HIPPO signaling [39]. The Hippo signal-mediated exercise therapy effect on CI is still unknown and needs to be further explored. There are limitations to this study. First, the KEGG enrichment analysis and pathway crosstalk analysis is based on genes from our RNA-Seq. CI is a continuous process disease and we just selected a signal pathway at a time node for analysis. Second, we excluded publications that may be related to CI.

Conclusion

In this study, we used a comprehensive and systematic biological function network analysis to explore CI-associated genes after exercise therapy from RNA-seq. Wnt signaling pathway, cAMP signaling pathway, calcium signaling pathway, Hippo signaling pathway, MAPK signaling pathway, cGMP-PKG signaling pathway were enriched in the CI gene set. Such exploration of the genes involved in exercise effects of CI will help us to identify potential biomarkers for further research.

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