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Predict Key Biomarkers of Alcohol Dependence in Nucleus Accumbens by Weighted Gene Co-Expression Network Analysis

Xugang Shi[1], Bing Xu[1], Taiping He[1], Jinghong Shi[1], Lijun Zhang[1]*

ABSTRACT

Alcohol dependence (AD) is a complication behavioral disease interacted by a number of genes and environmental factors with high heritability. The main point of the study was to identify the regulatory mechanism exerted by the nucleus accumbens (NAc) in AD. We not only performed DEGs analysis but also carried WGCNA analysis in GSE62699 dataset, and then we used online tools for gene enrichment analysis of the DEGs and hub genes, and explored possible transcription factors. We conducted DEG analysis first, and then opted the top 25% of genes with the maximum variance to perform WGCNA analysis. Finally, through GO and KEGG analysis, we found that acute inflammation is the most important biological pathway in DEGs. Then signal transduction and cell communication are the main results of BP in WGCNA. We used the hub gene to explore the transcription factor (TF) which may have a key role using the web tool--miRNet, and found that TP53 and NFKB1 are the important TFs in NAc. From the result, the miRNAs and TFs may serve as potential biomarkers and treatment targets of AD, and all these findings may be a theoretical basis to explore the regulatory mechanisms of AD.

INTRODUCTION

Known as a major global public health issue, alcohol consumption is an important risk factor for many health problems which represent an enormous health, social and economic burden worldwide Rehm et al. (2011). According to the World Health Organization's (WHO) 2016 Global Survey, 2.3 billion people over 15 years of age are drinkers. In the three WHO regions alone (the Americas, Europe and the Western Pacific), more than half of the population drinks alcohol (Organization, (2018). Alcohol dependence (AD) is a common, chronic relapsing and debilitating complex disorder which is a major risk factor for many non-communicable diseases Jones et al. (2008). Numerous post-mortem and in vivo neuroimaging studies in alcohol-dependent have proved that the alcohol toxic effects are particularly obvious in the brain Chanraud et al. (2007), Kril et al. (1997), Moselhy et al. (2001). Structural changes including gray-white atrophy, dilated sulcus and ventriculomegaly enlargement are clearly inspected in the brain. In addition, long-term alcohol consumption is along with neural adaptations in different neurotransmitter systems, such as the dopamine system Charlet et al. (2013), Spanagel et al. (2009). Lots of scientific research has proved that the etiology of AD is mainly manifested in the complex interaction between environment and genes Reilly et al. (2017). Some researchers performed a meta--analysis and found that the hereditary susceptibility of AD is 49%, which belongs to moderately heritable Verhulst et al. (2015).

In the central nervous system (CNS), there are some and complex neuronal nuclei and neural pathways associated with substance addiction and relapse. Among these pathways, the mesolimbic dopamine system (MLDS) is not only an important part of the brain reward system but also the most closely regulated pathway related to addiction and relapse. As an important structure in the MLDS, nucleus accumbens (NAc) is an extension of the ventral striatum, belonging to the basal nucleus of the forebrain. As a key part of the reward system, NAc plays a pivotal role in participating in drug addictive behavior, regulating the integration and expression of motivation and emotional activities Goto et al. (2008). and NAc circuits can be destroyed when exposed to drugs of abuse Russo et al. (2010). In the NAc, more than 90% of the principal cells are GABAergic medium spiny neurons (MSNs) Kreitzer et al. (2009), Zahm et al. (2000). MSN is divided into two types according to the expression of D1 or D2 dopamine receptors. The changes of D1+ and D2+MSN activity have different consequences for motivated and reward-related behaviors in NAc. When MSNs are at a very negative membrane potential and suddenly driven by excitatory inputs from other brain regions, the ventral hippocampus (vHPC) provides a powerful input that preferentially targets the medial shell of the NAc Britt et al. (2012) and the inputs are strongest

[1]Department of Medical Techniques, Shaanxi University of Chinese Medicine, Xianyang, Shannxi Province, PR China.

Correspondence to: Xugang Shi, Department of Medical Techniques, Shaanxi University of Chinese Medicine, Xianyang, Address: No.1, Middle Section of Century Avenue, Xixian New Area, Shannxi Province, PR China. E-mail: saintxugang@163.com,

Keywords: Alcohol dependence, Weighted gene co-expression network analysis, NAc, hub genes.

on the D1+MSNs which preferentially trigger action potentials (AP) MacAskill et al. (2014). However, there are few descriptions about the regulation or expression mechanism of the brain region, and further research is needed to explore the mechanism.

The present study aimed to apply weighted gene co-expression network analysis (WGCNA), a systems biology approach for identifying the key genes and miRNAs in significant modules as candidate biomarkers or therapeutic targets, on differentially expressed genes in NAc. The microarray datasets (GSE62699) which consist of samples with alcohol dependence (AD) and matched controls from frozen human post-mortem NAc tissues, stored in the Gene Expression Omnibus (GEO) database. We performed WGCNA to exploe novel biomarkers and provide some new perspectives for the molecular mechanism of AD in NAc. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were further performed to predict biological mechanisms of the hub genes in the key module. In addition, miRNet--an online tool, was utilized to investigate potential association molecules in AD.

METHODS

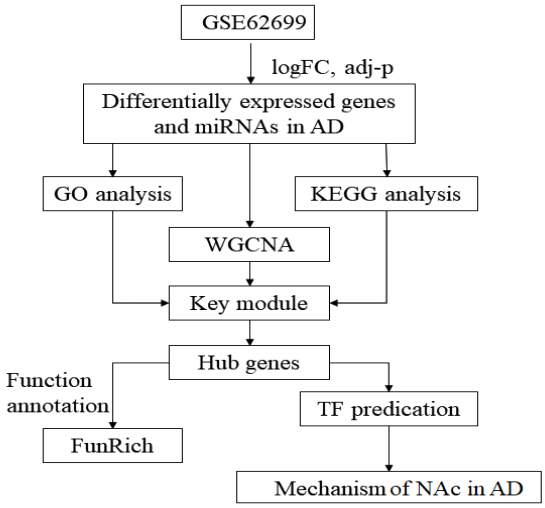
Data download

All microarray datasets were obtained from GEO datasets (www.ncbi.nlm.nih.gov/geo/). Based on the terms "Nucleus Accumbens", "NAc", "Alcohol dependence" and "AD", we finally selected the dataset: GSE62699 Mamdani et al. (2015). The GSE62699 dataset included two kinds of experiment data: Expression profiling, Non-coding RNA profiling. The NAc tissue samples in each experiment type of GSE62699 were composed by 18 patients and 18 matched controls. The miRNA and mRNA expression levels in case-control pairs with samples RINs ≥6 was judged on the Affymetrix GeneChip® Human Genome U133A 2.0 (HG-U133A 2.0) and Affymetrix GeneChip miRNA 3.0 microarray. All samples of the dataset came from the New South Wales Tissue Resource Centre. According to the laboratory's case exclusion criteria to select eligible cases: 1) infectious disease history (HIV/AIDS, hepatitis B or C etc.), 2) an unsatisfactory deathbed status (depended on the environment at the time of death), 3) a post-mortem interval > 48 hours, 4) significant head injury.

Differential gene expression analysis

Figure 1 exhibits the workflow for the identification and functional analysis of DEGs and miRNAs. The clinical data of GSE62699 only includes grouping. We manipulate ActivePerl (version 5.26.3; http://www.activestate.com/products/activeperl) to transfer the probe to the gene ID in the original data, and sort out the expression matrix based on the case-control grouping. Subsequently, we utilized the R package "limma" Ritchie et al. (2015) to normalize the data and and obtain the DEGs. Through adjusting threshold of logFC (Fold change) and adjust-P, we gained the most significant DEGs in the dataset.

Figure 1: Research workflow.



GEO, Gene Expression Omnibus; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; WGCNA, Weighted Gene Co-expression Network Analysis; NAc, nucleus accumbens.

Function enrichment analyses

We launched GO enrichment analysis and KEGG pathway analyses using FunRich3.1.3 and the R package "clusterprofiler" Yu et al. (2012). The adjusted $p < 0.05$ was considered statistically significant and GO terms or KEGG pathways with visualized by R package--"GOplot" Walter et al. (2015).

WGCNA

When the number of genes exceeds 5000, we extracted one-quarter of the DEGs, and the variance of the selected genes is greater than all the variance quartiles. WGCNA was performed using R/Bioconductor to find the clinical traits-related key modules and hub genes among them Langfelder et al. (2008). The pick Soft Threshold function was applied to calculate scale-free topology fitting index R2 that corresponding to different soft thresholding powers. We selected suitable power value as the soft threshold, and converted the gene expression matrix into an adjacency matrix and a Topological Overlap Matrix (TOM, TOMtype= 'signed'). Based calculated the corresponding dissimilarity of TOM value (dissTOM), genes were clustered into different gene modules. As the first principal component of gene expression for each module, module eigengene (ME) is considered as a representative and inter-relatedness of each module by eigengene network clustering. MEs were compared with

demographic data using Pearman's correlation corrected for clinical traits, and p <0.05 were applied for significantly.

Enrichment analysis for biological function of Modules

The biological function of modules was explored using "clusterProfilter" to determine. We selected the highest correlation module based on gene significance (GS) and module membership (MM), then performed the exploration of GO and KEGG.

miRNA correlation analysis

18 AD patients and 18 matched controls in GSE62699 were used to determine microRNA (miRNA) association. MEs were correlated to the miRNAs in the dataset using Pearson's correlation and p values<0.05 were considered significant. The enrichment analysis for biological function associated with miRNAs for the most significant module were identified using the software--FunRich Pathan et al. (2015).

Exploration analysis for transcription factors and miRNAs related pathway

We combined the most significant modules in the two kinds data, and utilized the online tools- miRNet (https://www.mirnet.ca/miRNet/faces/home.xhtml) to explore the TF (transcription factors). The online miRNAs Enrich tools- miEAA (https://ccb-compute2.cs.uni-saarland.de/mieaa2/) used to explore the miRNAs related pathways.

RESULTS

Differential gene and miRNA expression analysis

After performing gene ID conversion and clinical group distinction on the expression matrix, we adjusted the thresholds of logFC and adj-P appropriately according to the demands of analysis. Small differences in the nervous system can cause huge effects in the human body. When logFC > 1 and adj-P<0.05 in GSE62699 dataset, 85 up-regulated and 29 down-regulated significant DEGs were calculated out. And when logFC > 1, the miRNA expression matrix only found three miRNAs: hsa-miR-34c-3p, hsa-miR-34b, hsa-miR-34c-5p. The up-regulated and down-regulated DEGs and miRNAs are shown in Table 1 and Figure 2.

Figure 2: Heatmaps of DEGs in NAc.

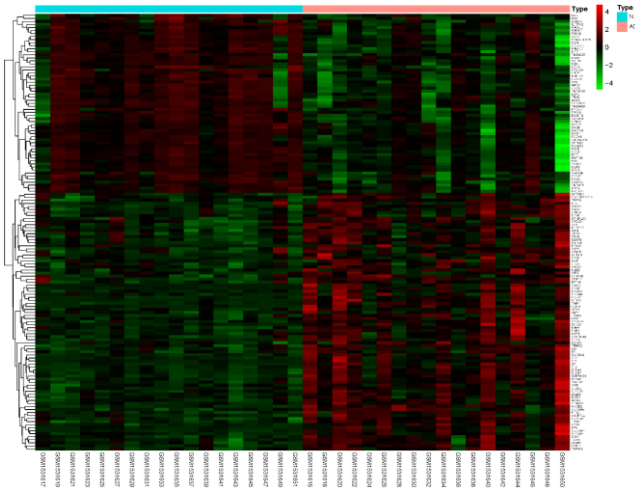


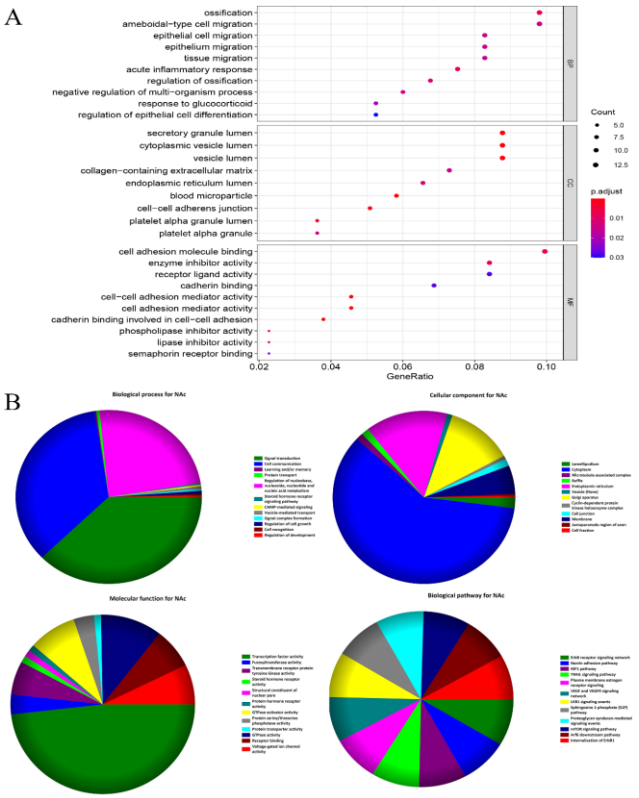
Table 1: List of differentially expressed genes and miRNAs in two datasets.

ID	Tissue	Classification	Upregulated	downregulated
GSE62699	NAc	gene	SERPINA3, CP, ANXA1, SLC14A1, SLCO4A1, PLA1A, ANXA2, TXNIP, ANGPTL4, TUBB6, MAFF, EDN1, PDLIM1, IFITM2, TIMP1, IFITM3, ZBTB16, NTS, CFH, ACSL5, APLNR, SERPING1, VSIG4, RFTN1, IGFBP3, CLEC2B, ZFP36, C1R, SLC16A6, SPAG6, IFITM1, VWF, ECM2, CLIC1, S100A8, APOLD1, HILPDA, MTHFD2, SRGN, TPPP3, ID3, SLC26A2, MGP, HSPB1, ATF3, CTGF, SRPX, TRIM22, GEM, MAFB, JUN, ACKR3, C1S, CFI, ELTD1, CD163, CEBPD, FSTL1, SLC16A1, IL1RL1, MOXD1, EMP1, CXCL1, CDKN1A, ICAM2, LOC100272216, MT1X, CRISPLD2, CLDN5, GBP1, C10orf10, AQP1, BAG3, TGIF1, S100A11, ID1, KCNK10, TAGLN2, PTGS2, IGF2BP2, HMGB2, ANXA3, TSPAN6, STON1, SOX9	GLRB, RNF128, TMEM35, DYNC1I1, MAD2L1, DHCR24, DSP, EPCAM, MAN1A1, PRKCH, IL13RA2, GABRG2, HN1, ITFG1, MYOZ3, AOAH, NUDT11, DGKB, MAGEL2, PCDH8, MST4, GLCE, TUSC3, HCG4, SORCS3, SEMA3A, SCG2, CCDC88C, CACNA2D3, B3GNT2, CHGB, STAT4, ACTL6B, NIT2, CDH12, LMO7, FAT2, TMEM257, TMEM159, ST6GALNAC5, ACOT7, NMNAT2, CRYM, TMEM45A, RIT2, NSG1, TRAC, SEMA3E, PEG10, SAG, GUCA1A, SMCO4, HTR2C, PENK, CALB1, OCA2, DIRAS3, IMPG1, PDYN
		miRNA	hsa-miR-34c-3p, hsa-miR-34b-star, hsa-miR-34c-5p	

Functional enrichment analysis of DEGs and miRNAs

All of the DEGs were chosen to run GO and KEGG analyses. From the results, we found the DEGs in NAc are enriched in several biological process (BP): acute inflammatory response, ossification, ameboidal−type cell migration; In terms of cellular component(CC), collagen−containing extracellular matrix, cytoplasmic vesicle lumen et.al were enriched; Some molecular function(MF) GO terms, such as cell adhesion molecule binding, receptor ligand activity, cadherin binding were enriched (Figure 3A); In the KEGG pathway analysis, only one result --- complement and coagulation cascades mostly associated with these genes. In the miRNA analysis of NAc, signal transduction, cell communication and learning and memory is the most association BP; lamellipodium is the most association CC; transcription factor activity in terms of MF was the most significantly enriched GO term; As to KEGG pathway analysis, ErbB receptor signaling network, nectin adhesion pathway, IGF1 pathway, TRAIL signaling path, Plasma membrane estrogen receptor signaling were mostly associated with the miRNAs (Figure 3B).

Figure 3. GO and KEGG analysis of DEGs.



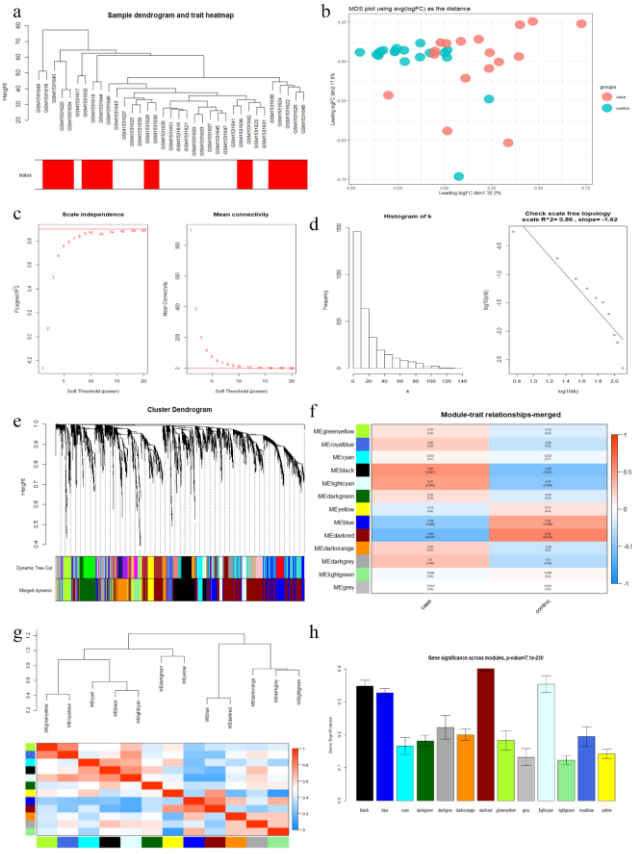
(A) Bubble plot depicting the relationship between NAc genes and GO terms of biological process (BP), cellular component (CC), and molecular function (MF).

(B) Pie plot depicting the relationship between NAc miRNAs and GO terms of BP, CC, MF, Biological pathway.

WGCNA module identification

We performed three WGCNA on the genes of NAc and miRNAs of NAc to find the key modules which is most association with AD (Figure 4-5). We first optioned the top 25% of genes with the maximum variance as the input dataset to conduct WGCNA analysis. Then based on the clustering results of gene and miRNA expression, we deleted the outliers. The samples size of the NAc gene set and miRNA expression set are 35 (18 controls: 17cases) and 32 (16controls:16cases), respectively. We finally identified 13 modules (Gray module displayed non-clustering DEGs) in NAc gene matrix through the soft-thresholding power as 9 (scale free R2 = 0.86) and cut height as 0.35. From the heatmap of module–trait correlations, we identified that the black module was the most highly correlated with AD patients. Figure 5 indicated that through the soft-thresholding power as 5 (scale free R2 = 0.97) and cut height as 0.35, we finally identified 5 modules in NAc miRNA expression matrix. The turquoise module was the most association with case in the data.

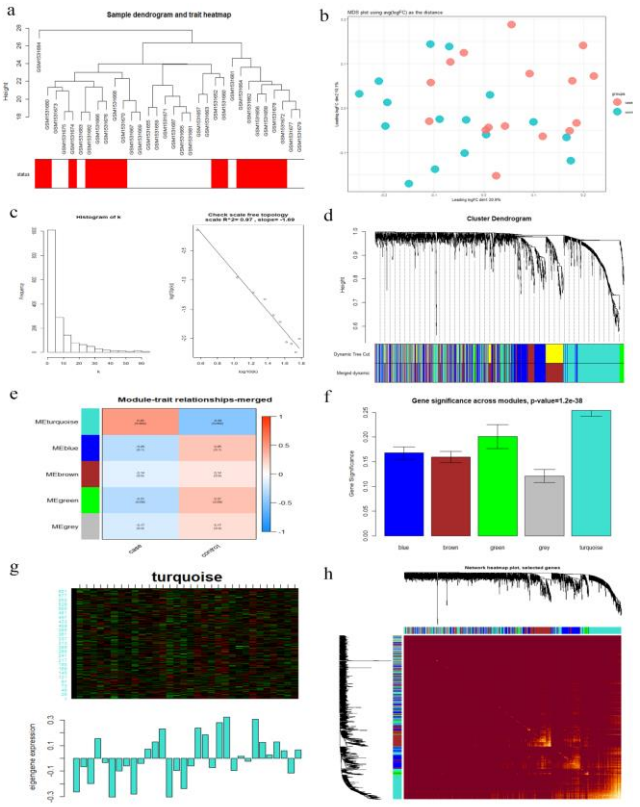
Figure. 4. Identification of key gene modules correlated with clinical traits in the GSE62699 dataset through WGCNA.



(A) Clustering dendrograms of genes. The clustering was based on the top 25% expression data of GSE62699. Color intensity varies positively with status. In terms of biochemical recurrence, red means recurrence and white indicates no recurrence.

(B) MDS (Multidimensional Scaling) clustering on the cut samples. (C) Analysis of the scale-free fit index (left) and the mean connectivity (right) for various soft-thresholding powers. (D) Check whether the memory network is close to scale free under the selected β value. (E) Dendrogram of all DEGs clustered based on a dissimilarity measure (1-TOM). (F) Heatmap of the correlation between module eigengenes and clinical traits of AD. Each group contains the correlation coefficient and P value. (G) Relationship between traits and modules. (H) Distribution of average gene significance and errors in the modules associated with Gleason score of AD.

Figure 5: Identification of key miRNA modules correlated with clinical traits in the GSE62699 dataset through WGCNA.



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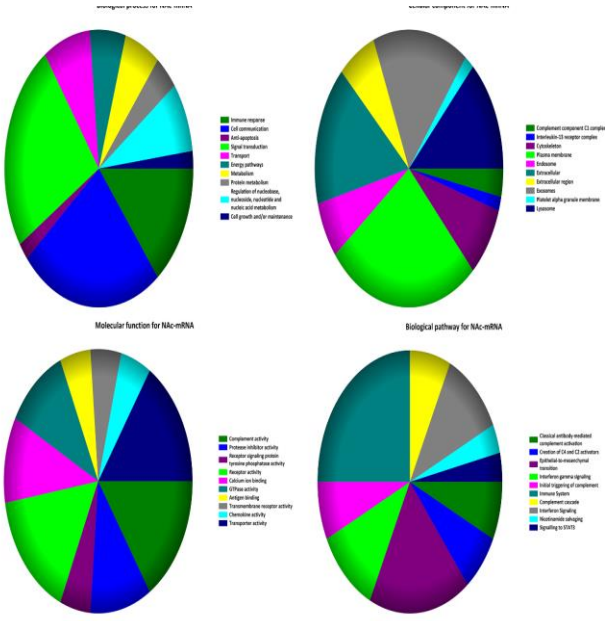
Hub genes obtain and pathway enrichment analysis

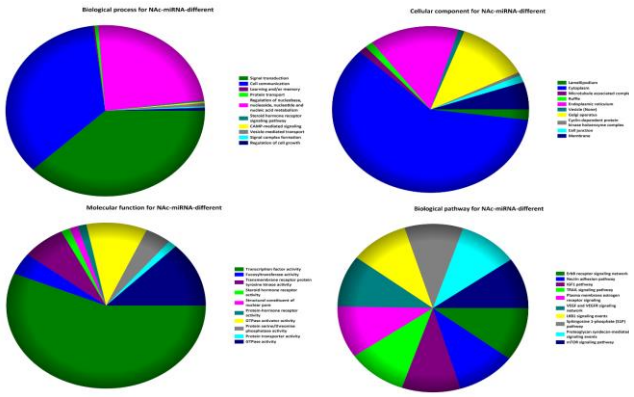
Hub genes are those that display the highest connectivity in the network. Output the KME (eigengene connectivity) matrix of all genes and modules, and then filter the first few genes with the largest KME in each module as hub genes. The hub genes and miRNAs from the key modules are listed in Table 2. Setting module membership (MM) >0.85 in NAc gene matrix, we selected 37 hub genes from 346 genes of the black module. We performed GO and KEGG analyses in FunRich to explore potential biological functions of the hub genes within the black module.

The results were listed in Figure 6A and stated that genes were mainly involved in signal transduction, cell communication and immune response. From the NAc miRNA turquoise module, we selected 32 hub miRNAs (MM >0.9) to perform the miRNA enrichment analysis. From the results of Figure 6B, we found the miRNAs were mainly participated in signal transduction, cell communication and regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism. Meanwhile, we have obtained some metabolic pathways that may play a pivotal role in the pathogenesis of AD from the online tools- miEAA, such as regulation of extrinsic apoptotic signaling pathway via death domain receptors (GO1902041), activation of MAPKK activity (GO0000186), and so on (Figure S1).

Figure 6: GO and KEGG analysis of hub genes in GSE62699 through FunRich.



(A) Pie plot depicting the relationship between NAc genes and GO terms of biological process (BP), cellular component (CC), and molecular function (MF) and biological pathway.



(B) Pie plot depicting the relationship between NAc miRNAs and GO terms of BP, CC, MF, Biological pathway.

Prediction of TFs and miRNAs enrichment analysis

Based the respective genes and miRNAs in their modules, we conducted the TF prediction using the online tool – miRNet. From the genes and miRNAs result of NAc (Figure 7A, 7B),

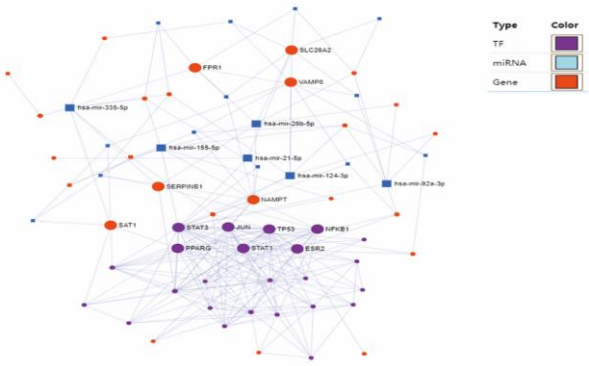
we found that TP53 (tumor protein p53) and NFKB1 (nuclear factor kappa B subunit 1) are the most important TF in NAc.

And we found that several miRNAs also nave key roles involved in AD (has-miR-155-5p, has-miR-26b-5p, has-miR-92a-3p and has-miR-124-3p.

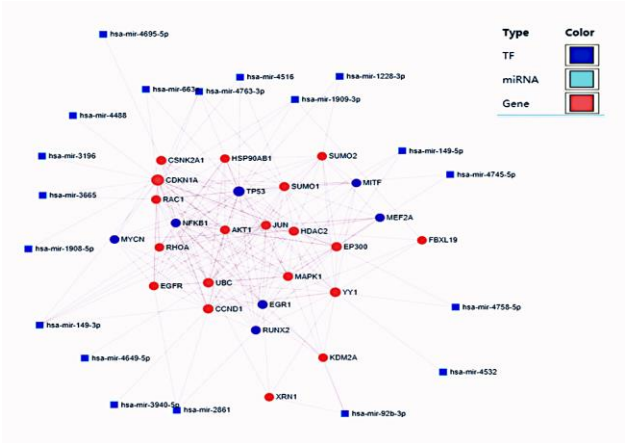
Table 2: List of hub genes and miRNAs in the dataset.

ID	Tissue	Classification	KME (Keymodule)	hub moleculars
GSE62699	NAc	gene	>0.85(black)	SQRDL, CD93, PLSCR1, FPR1, S100A11, VSIG4, C1QB, DSE, SERPINB1, STAT3, NAMPT, YBX3, PECAM1, C1QA, CD163, HCLS1, SAT1, MTHFD2, GBP1, SLC26A2, PTPRC, FCER1G, GBP2, TNFRSF1B, MAFB, FCGR1B, MT1M, IL13RA1, FAM129A, EMP1, PDLIM1, VAMP8, MYL12A, ELF1, MT1X, SERPINA1, CMTM6
		miRNA	>0.9(turquoise)	hsa-miR-4466, hsa-miR-4745-5p, hsa-miR-3940-5p, hsa-miR-3656, hsa-miR-3960,
				hsa-miR-4695-5p, hsa-miR-2861, hsa-miR-4488, hsa-miR-663, hsa-miR-4687-3p,
				hsa-miR-1469, hsa-miR-1908, hsa-miR-149-star, hsa-miR-1909, hsa-miR-1915,
				hsa-miR-4463, hsa-miR-1228-star, hsa-miR-3665, hsa-miR-4707-5p, hsa-miR-4516,
				hsa-miR-1268, hsa-miR-4787-5p, hsa-miR-3648, hsa-miR-3621, hsa-miR-4649-5p,
				hsa-miR-3196, hsa-miR-4532, hsa-miR-4763-3p, hsa-miR-4497, hsa-miR-4734,
				hsa-miR-4758-5p, hsa-miR-92b-star

Figure 7: Correlation among hub genes, miRNAs and TFs in the datasets.



(A) Depicting the relationship of NAc hub genes in the miRNet analysis.



(B) Depicting the relationship of NAc hub miRNAs in the miRNet analysis.

DISCUSSION

Combining differential expression analysis and WGCNA, our goal is to explore the candidate key molecules and pathways of AD in human NAc. The research results explored the key TFs that affect AD in the NAc. By further research the function of TFs which regulate gene transcription and regulation, change signal pathway transduction, and affect downstream gene expression, we hope to guide further research on the molecular mechanisms and functions of AD.

AD is a serious psychiatric disorder that manifested by a strong desire to drink, loss of control over drinking and excessive alcohol consumption, in which the changes of various neurotransmitters and their receptors in specific areas such as the reward circuit plays a momentous role in the development of addiction Gilpin et al. (2008). The reward circuit of brain includes a lot of brain area. Among them, NAc is the core brain region that produces all reward effects. From the literature of Tara A. LeGates1, they summed up the viewpoint the strength of hippocampus-nucleus accumbens synapses is regulating reward behavior LeGates et al. (2018). In our study, we explored the association between NAc and AD through the DEGs and WGCNA. Based our results, we figured out the distinctive mechanism and key role of NAc in AD.

The NF-ϰB/Rel family contains five members: p65 (RelA), p50/105 (NFKB1), p52/p100 (NFKB2), c-Re1 and RelB. NFKB1 is located on chromosome 4q24 and encodes two proteins, non-DNA binding protein p105 and DNA binding protein p50. P50 is equivalent to the N-terminus of p105, which functions by binding to DNA through p50 / p50 homodimers or p50 / p65heterodimers Eskandari-Nasab et al. (2016), Loganathan et al., (2013). The p50/p65 heterodimer, also known as "NF-ϰB", is the most common and abundant dimer in the human body. It plays pro-inflammatory properties by increasing the transcription of inflammatory cytokines, such as TNF-α and interleukin (IL)-12. The p50/p50 homodimer exerts anti-inflammatory effects by stimulating the transcription of anti-inflammatory cytokines, such as IL-10 Chen et al. (2016). Long-term drinking alcohol is a risk factor for the development of alcohol dependence (AD) and is known to modulate the immune system in a complex manner Crews et al. (2006). TNF-α activates resident microglial cells, induces neuro-inflammation, inhibits glutamate transporters, changes glutamate receptors, and leads to high glutamate expression. Too high glutamate will increase alcohol intake, inhibit neurogenesis and accelerate neuronal apoptosis, thereby promoting AD Spanagel et al. (2005). Our result found the NFKB1 is the one of the important TFs in GSE62699, and from the tool- TFactS (http://www.tfacts.org/TFactS new/TFactSv2/index1.html) also demonstrated it is an important TF which plays regulatory roles in the DEGs of NAc. The activity of NFB1 may be triggered by long-term alcohol exposure, and after the activated NF-ϰB enters the nucleus, it initiates the transcription process, induces the expression of a large number of inflammatory factors such as TNF-α, causes the high expression of glutamate which leads to the occurrence of AD.

MiRNAs are a group of highly conserved non-coding RNAs which length about 20-22 nucleotide and regulate gene expression through binding to 3'UTR of target mRNAs (Bartel et al. (2009). Not only can miRNA degrade targeted transcripts or obstruct the translation of encoded proteins, but also can stimulate translation under certain special conditions. Has-miR-124 is one of the most natural miRNAs settled in the nervous system and plays effective functions in many fields of CNS, such as neuronal development and neural plasticity Mucaj et al. (2015). In different studies, the researchers found morphine or nicotine significantly upregulate miR-124 expression through different cell and receptors. In the same research of morphine, NF-ϰB p65 direct combines with the promoters of pri-miR-124-1 and pri-miR-124-3 in mice which to product the more miR-124 and also found c-Fo's overexpression in BV2 microglial cells induces the expression level of miR-124 decreases. In the research, TFs are the mainly regulator of miR-124 expression changes, such as c-Fos Qiu et al. (2015). In our results, we found the TF –JUN, which also known as AP-1 (activator protein 1) transcription factor subunit, encodes the protein which is highly similar to the viral protein that directly interact with paticular target DNA sequences to regulate gene expression. Numerous miRNAs regulate inflammation pathways, such as NF-ϰB, STAT3, AP-1 signaling. The study of Wang explained that PPARγ (Peroxisome proliferator-activated receptor gamma) was mediated by the up-regulation of miR-124 by binding to the miR-124 promoter region, and inhibited AP-1 and NF-ϰB plays an anti-inflammatory role in disease Wang et al. (2017). The above study gives us a revelation that the suppression of transcription factors and the regulation of miRNAs may be together promote the formation of AD. As an important data analysis tool, WGCNA is widely applied for kinds of diseases. DEGs analysis and WGCNA analysis were both performed in the GSE62699 by us. We extracted the hub genes in the most relevant modules of the case group for function and TF exploration in AD. Our results explored the pathways and key TFs that may appear in NAc during the formation of AD.

CONCLUSION

Through careful explore of bioinformatics databases, our results theoretically reveal the relevant regulatory pathway and molecules in NAc that play a key role in AD,such as cell communication and signal transduction, and found some key biomarkers such as TFs: TP53,

NFKB1; miRNA: has-miR-155, has-miR-26b-5p, has-miR-92a-3p and has-miR-124-3p. One limitation of the study is the sample size is relatively small. Another is the biases inherent in existing clinical data and enrichment analysis. Because it is based on a mature verified list of genes, it only may be reflexed in the number of immune response modules that have been identified. We need to conduct functional verification and model animal experiments to further verify the role of our results and determine their functional significance in AD progression.

Abbreviations

GEO: Gene Expression Omnibus

AD: Alcohol dependence

NAc: Nucleus accumbens

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

WGCNA: weighted gene co-expression network analysis

GS: gene significance

MM: module membership

DEG: differentially expressed gene

CNS: central nervous system

DECLARATIONS

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Availability of data and material

The datasets analyzed during the current study are available in the GEO datasets (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62699). The data can be downloaded using R software.

Authors' contributions

XG S designed the study, performed data analysis and revised the images; TP H, LJ Z, and JH S assisted with data analysis and interpretation of findings. B X revised the manuscript. All authors reviewed and approved the manuscript.

Competing interests

All authors declare that there is no conflict of interest regarding the publication of this article.

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