

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Systematic Review

Comprehensive Analysis of the Gene Expression Profiles of Rat Brain Tissues under Environmental Exposure to Nicotine

^{1,2}Wuyi Liu, ¹Huifang Lv, ¹You Zhou, ¹Xiang Zuo and ¹Xin Wang

¹Department of Biological Science, School of Biological Science and Food Engineering, Fuyang Normal University, Fuyang, China

²Anhui Engineering Technology Research Center of Anti-Aging Chinese Herbal Medicine, Fuyang Normal University, Fuyang, China

Abstract

Background and Objective: Nicotine-relevant smoking causes many serious issues of environmental pollution and complicated harm to human health. The present study aimed to evaluate the experimental effects of exposure to nicotine on the gene expression profiles of rat brain tissues with differentially expressed genes (DEGs). **Materials and Methods:** The rat gene expression profiles of environmental exposure to nicotine were initially screened and retrieved from the microarray dataset GSE59895 in the GEO database. Next, it was analyzed with an integrated bioinformatics pipeline. The DEGs were analyzed in Limma and functional enrichment analyses of GO terms and KEGG pathways were performed with clusterProfiler. The STRING online tools and Cytoscape StringApp were subsequently employed to construct the protein-protein interaction (PPI) network, whereas key modules and hub genes were finally explored and visualized. **Results:** There was total of 382 shared DEGs between different case groups in the experiment, whereas 9 common shared DEGs were found among all three groups. The significant enrichments of 28 GO terms and 3 KEGG pathways were comprehensively analyzed with corresponding functionally enriched genes. Then, 3 key modules and 10 hub genes were further identified and explored in the resulted PPI network. In the disease-related signaling pathways, eleven potential neuropathic disease-related genes may complement the treatment of neurodegenerative diseases. **Conclusion:** The study found that chronic exposure to nicotine would result in the differential expression of the disease-related genes, whereas these DEGs might increase the environmental risks of Huntington's disease, Alzheimer's disease and other multiple neurodegenerative diseases.

Key words: Nicotine exposure, brain tissue, rat brain, differentially expressed genes, neurodegenerative disease

Citation: Liu, W., H. Lv, Y. Zhou, X. Zuo and X. Wang, 2024. Comprehensive analysis of the gene expression profiles of rat brain tissues under environmental exposure to nicotine. Pak. J. Biol. Sci., 27: 547-566.

Corresponding Author: Wuyi Liu, Department of Biological Science, School of Biological Science and Food Engineering, Fuyang Normal University, Fuyang, China

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Smoking causes many serious environmental pollution issues, including increased industrial and ecological contaminations, human cancers and other physiological and mental menaces, as well as many socio-economic problems¹⁻⁴. The harm of smoking to the public environment is obvious and it is one of the biggest public health problems that continue to threaten human health in the world¹⁻⁶. Smoking a single cigarette may destroy the air quality 10 times or so in merely 5 min. In a relatively enclosed room, no matter how people increase their physical distance from smokers, they cannot avoid the passive absorption of secondhand smoke^{1,7-13}. Furthermore, the smoke fog produced by smoking contains some pathogenic substances such as nicotine, nitrogen dioxide, hydrocyanic acid, acrolein, arsenic and even lead and mercury^{4,6,11,12}. The concentration of various pathogenic substances in the smoke fog have exceeded the industrial permit threshold, which is the highest concentration of harmful gases stipulated by workers in advanced industrial countries. The harm of cigarette smoke to the population exceeds that of ordinary chemical gases in the industry. The mixed smoke fog of nicotine, tar, carbon monoxide, benzopyrene, aldehyde, cyanide and other harmful substances is mainly produced during the burning of cigarettes^{11,12}. When people smoke, the mixed smoke fog is expelled into the air with pathogenic substances like nicotine^{5,6,14} and nitrogen dioxide^{11,12}. In addition, the cigarette butts produced by smoking are non-biodegradable garbage, which will not only corrode the surrounding land but also greatly reduce the ability of the land to retain water and fertilizer. The cigarette butts in the earth deteriorate the water quality and cause inconvenience to the utilization of water resources too. Because cigarette butts are non-biodegradable garbage, it is an integral part of the environmental pollution caused by smoking, including second-hand smoking or passive smoking^{2,7,8,10,13}.

Smoking, including active or passive smoking, is a complex individual behavior involving the interaction of genetic, physiological and environmental factors^{10,15-18}. ASAE crucial component of tobacco and nicotine can cause people to become dependent on tobacco by acting on acetylcholine receptors in the central nervous system, i.e., nicotine dependence (or smoking addiction). It is an indisputable fact that smoking is harmful to health and smoking may lead to lung cancer. Millions of people die from lung cancer every year and more than 85% of lung cancers are related to smoking^{7,17,18-28}. The incidence of lung cancer ranks first in the occurrences of malignant tumors in many countries.

Long-term smoking is an important factor leading to lung cancer. However, with the continuously increasing air pollution, the attention of air quality in the environment is strongly strengthened. Severe environmental pollution is also considered to be another important risk factor of increasing the incidence and mortality of lung cancer^{11,29,30}. Recent studies have shown that long-term exposure to high concentrations of the inhalable particulates or particles in the air will increase people's risk of developing lung cancer^{1,7-9,11,12,31,32}. Some researchers made detailed statistics of the content of PM_{2.5} particles³³⁻³⁵. According to their statistical data in indoor research, more than 60 known carcinogens have been detected in the air environment where typical cigarettes can produce smoke³³⁻³⁵. For people who smoke for a long time and usually in heavily smoking-polluted environments, how do these two factors "combine" to increase people's risk of lung cancer? How do smoking and its surrounding environmental pollution have an impact on the morbidity and mortality of lung cancer? Despite increasing genetic evidence of smoking-attributable diseases, the detailed genotoxic pathways and susceptibility genetic regulatory mechanisms underlying various smoking behaviors are largely unclear. So far, genetic association studies have revealed numerous gene variants underlying smoking-attributable diseases. At present, the most extensively investigated susceptibility genes are the studied various gene members of the Nicotinic Acetylcholine Receptor (nAChR) gene family, including α 2- α 7 and β 2- β 4^{4,5,36,37}.

Nicotine is not only the main addictive compound causing smokers to continue their smoking behaviors but also a genotoxic contributor to the pathogenesis of cancers^{29,30}. With increasing genetic evidence, it is revealed that nicotine is a tertiary amine alkaloid believed to be the major enhancer and addictive ingredient of tobacco smoked^{21,22,38-40}, although the susceptibility genes and genetic loci of smoking addiction are unclear yet. Biological targets of nicotine action are different members of the neurotransmitter-gated ion channel superfamily of the Nicotine Acetylcholine Receptors (nAChRs)^{21,22,39,41}. The nAChR molecule is a kind of transmembrane allosteric protein that mediates transduction of chemoelectric signals throughout the nervous system by opening an intrinsic ionic channel^{30,42}. Nicotine and its receptors (nAChRs) play an important role in physiological function and pathological processes by mediating a variety of physiological processes and cellular signaling pathways^{30,42}. In physiology, nAChRs are responsible for muscle contraction, nerve transmission, sensory transmission, etc. Meanwhile, nAChRs are the key mediators of tobacco addiction and also important therapeutic targets for diseases in pharmacology.

The effects of exposure to nicotine mainly include impacts on the respiratory system, nervous system, digestive system and reproductive system in the human body to different degrees. Chronic exposure to nicotine is liable to cause neurological disorders, low immunity and immune system monitoring, defense and adjustment problems, resulting in neurological diseases, chronic cough, respiratory infection, oral mucosa, emphysema, gastrointestinal dysfunction, atherosclerotic plaques, heart head blood-vessel sclerosis, multiple sclerosis and primary biliary cirrhosis. Severe cases of exposure to nicotine could lead to lung cancer and even death^{2,9,10,16,18,20,22,30,31,39,40,43}. Among the identified susceptibility gene loci for smoking- attributable diseases, the most robust replicated findings in genome-wide association studies are those nAChR subunit variants in the CHRNA5/A3/B4 cluster on chromosome 15^{14,19,44-46}, CHRNB3/A6 on chromosome 8^{44,46} and CYP2A6/A7 on chromosome 19^{14,44,46}. Those nAChR subunit genes show a significant association with both nicotine dependence and lung cancer⁴⁴. However, the current genetics-based evidence is still lacking for elucidating the genotoxic pathways and carcinogenic mechanisms of cigarette smoking-associated diseases with gene expression profiles or shared functional expressed genes.

There are presently a few reports on the effects of environmental exposure to nicotine on the gene expression profiles in animal brain tissues. In the study, experimental samples were selected and retrieved from the GEO database in the National Center for Biotechnology Information (NCBI). An integrated bioinformatics pipeline was used to screen DEGs in the rat brain under the influence of nicotine. First, DEGs were analyzed with Limma⁴⁷ and clusterProfiler⁴⁸ in the rat brain tissues after exposure to nicotine. Next, it was projected to explore the crucial hub genes, signaling pathways, biological processes and molecular functions of these disease-related potential genes differentially regulated by exposure to nicotine. Functional enrichment analyses were performed on GO terms and KEGG pathways of the differentially expressed genes. The PPI network was finally constructed to figure out the key modules and hub genes with those DEGs and their biological function annotations. The study provided insights into the effects of environmental exposure to nicotine on the gene expression

profiles in rat brain tissues. The study may serve as a research foundation for further attempts at analyzing and exploring the molecular mechanism of diseases related to exposure of nicotine.

MATERIALS AND METHODS

Study duration and location: From 2021 to 2024, the study was conducted to evaluate the effects of exposure to nicotine on the gene expression profiles of rat brain tissues based on the retrieved and downloaded microarray dataset in Fuyang City, China.

Sample information: After screening in the GEO database in NCBI with the searching keywords “(*Rattus norvegicus*) and (Nicotine)”, the gene expression profiles of brain tissue were selected and retrieved from the GEO database within the microarray dataset GSE59895, which was initially uploaded by the researchers at the National Institute of Environmental Health Sciences¹. In their experiment, complete drug matrix datasets were designed for rat brains. Approximately 600 different compounds were profiled in up to 8 different rat tissues by obtaining tissue samples from test compound-treated and vehicle control-treated rats in biological triplicates for gene expression analysis after 0.25, 1, 3 and 5 days of exposure with daily dosing toxicants.

The GSE59895 dataset includes the following samples, i.e., 1 day of exposure to nicotine (including 1 case in the exposure group and 3 cases in the control group), 3 days (3 cases in the exposure group and 2 cases in the control group) and 5 days (3 cases in the exposure group and 3 cases in the control group) (Table 1). The microarray experimental platform is GPL5425 GE Healthcare/Amersham Biosciences Codelink Uniset RAT I Bioarray, Layout Exp5280X2-613.

Analyses of the experimental gene expression profiles:

The experimental gene expression profiles of rat brain tissues were analyzed with the integrated bioinformatics pipeline including Limma, clusterProfiler, StringApp, MCODE, CytoHubba, BINGO and the other software packages. The analysis was focused on the effects of the environmental exposure to nicotine.

Table 1: Sample information in the GSE59895 dataset

Treatment (day)	Case group of exposure to nicotine	Control group
1	GSM1450189	GSM145028, GSM1450281 and GSM1450282
3	GSM1450190, GSM1450191 and GSM1450192	GSM1450283 and GSM1450284
5	GSM1450193, GSM1450194 and GSM1450195	GSM1450285, GSM1450286 and GSM1450287

Identification of DEGs: The linear models of Limma version 3.44.3⁴⁷ were applied to analyze the difference in exposure to nicotine at day 1, 3 and 5, respectively. The Limma software mainly employs the following linear regression model to fit each gene's expression:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_p X_p + \varepsilon$$

In which ε is the residual term. The core R analysis code of the Limma package is briefly listed as follows, i.e., "design <- model.matrix(~0+factor(list))" and "df.matrix <- makeContrasts(nicotine-normal, levels = list)". The set parameters of adjusted $p < 0.05$ (or adj.p.val < 0.05) was adopted as the standard of identifying DEGs, while the ggplot2 version 3.3.3^{49,50} was used to map the volcanoes of identified DEGs. Herein, the Venny online tool was used to intersect the shared DEGs among three case groups of nicotine exposure. These shared DEGs were used for subsequent analyses.

Functional enrichment analyses of GO terms and KEGG pathways: The clusterProfiler version 3.16.1⁴⁸ was used for GO and KEGG analysis of shared DEGs. The adjusted $p < 0.05$ (or adj.p.val < 0.05) was considered biologically significant⁴⁸.

PPI network and identification of key modules and hub genes: The PPI network was constructed and visualized with all the annotated proteins available of the shared DEGs among different case groups of rat exposure to nicotine using the STRING online tool⁵¹ and the StringApp software in Cytoscape version 3.8.0⁵². The searching algorithm in the MCODE software⁵³ was employed to identify densely connected PPI modules of the shared DEGs with the following parameters of degree cutoff to 4, k-core to 4, max and depth to 100. The MMC algorithm of the CytoHubba software⁵⁴ was further applied to identify all the potential disease-related DEGs.

Subsequently, the BINGO software⁵⁵ was adopted in the functional enrichment analyses of GO terms and KEGG pathways in subnetworks (PPI modules) on the disease-related DEGs. The PPI network and subnetworks (PPI modules) were further explored and displayed by the other Cytoscape software and plugins.

RESULTS AND DISCUSSION

Assessment of experimental differential expression profiles and identification of the shared DEGs:

In the experiment, linear models in the Limma software⁴⁷ were applied to assess the rat differential expression profiles and there were 1721, 64 and 1218 DEGs identified in the three case groups of exposure to nicotine, respectively. Specifically, 1721 DEGs were identified in the first day of exposure to nicotine including 1056 up regulated genes and 665 down regulated genes, whereas 64 DEGs were identified in the third day of exposure to nicotine including 48 up regulated genes and 16 down regulated genes. There were 1218 DEGs identified in the 5 days of exposure to nicotine, among which 630 genes were up regulated and 588 genes were down regulated (Fig. 1). Meanwhile, there were many shared DEGs identified for the dealt trials of 1, 3 and 5 days of exposure to nicotine with the newest version of Venny online tool⁵⁰. Specifically, there were 20 shared DEGs identified for the dealt trials of 1 and 3 days of exposure to nicotine, 343 shared DEGs identified for 1 and 5 days of exposure to nicotine and 37 shared DEGs discovered for the dealt trials of 3 and 5 days of exposure to nicotine (Fig. 2). Furthermore, there were nine common shared DEGs among all the three groups, including Serpini1, RGD1565416, Slurp1, KB1, PGR, FRMD4A, PPP2R2C, Rph3A and HtrA3. Subsequently, there were a total of 382 shared DEGs identified between different case groups of exposure to nicotine.

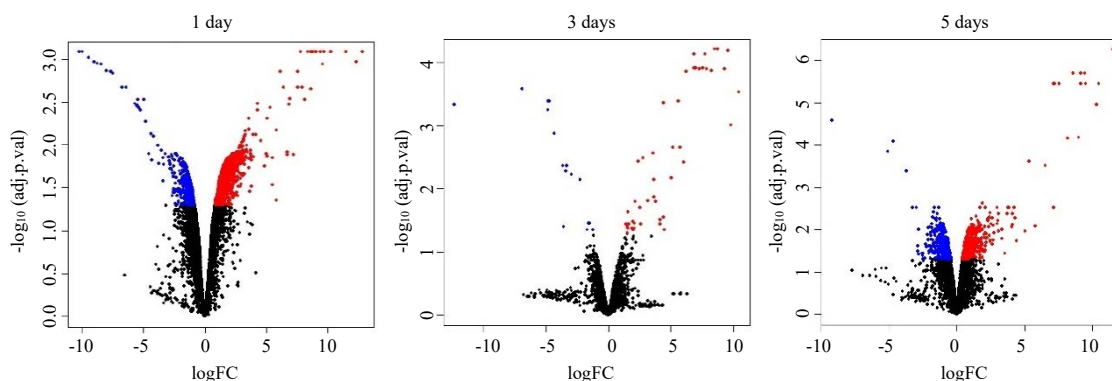


Fig. 1: Volcano maps of DEGs in different case groups

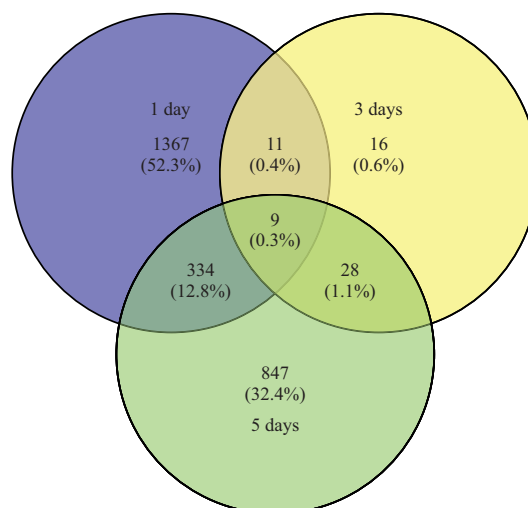


Fig. 2: Venn map of overlapping DEGs among different case groups

In the study, nine common shared DEGs (Serpini1, RGD1565416, Slurp1, KB1, PGR, FRMD4A, PPP2R2C, Rph3A and HtrA3) were discussed with interest.

Serpini1: Mammalian serpin family I member 1 (Serpini1) is also known as HNS-S1, HNS-S2, PI12 and neuroserpin. Rat Slurp1 is also named as the PI-12 and raPIT5a. Neuroserpin (or Serpini1) is a neurogenic serine protease inhibitor involved in the formation and connection of nerve cells and the plasticity of synapses in the adult nervous system⁵⁶⁻⁶⁰. Neuroserpin (or Serpini1) is the tissue specific inhibitor and the tissue-type plasminogen activator in physiological processes^{56,57,61-66}. They play a role in synaptic plasticity, memory or sterol metabolism in normal physiological environments^{56,61,62}, while they can also induce Alzheimer's disease in pathological environments⁵⁷⁻⁶³. Although neuroserpin is expressed during late stage of neurogenesis in development, it was suggested that neuroserpin might played some roles in early stage of neurogenesis in adult rat hippocampus⁶⁷. As an inhibitor of extracellular plasminogen activator in the central nervous system, neuroserpin regulates the hydrolytic activity of fibrinolytic enzyme and plays roles in blood coagulation and fibrinolysis and diseases⁶⁸. Neurofilamase inhibitory protein was reported with a protective effect on the ischemic nervous system⁶⁹, which agrees well with the enriched GO term (GO:0002931) in this study. Short-term exposure to nicotine among non-smokers significantly up-regulates the secretion level of neuroserpin (or Serpini1)⁵⁸, which participate in the synaptic plasticity process. Conversely, abnormal expression of neuroserpin (or Serpini1) may be associated with the smoking-related neurodegenerative diseases⁵⁹.

RGD1565416: The protein expression of RGD1565416 is also known as talin 2 (Tln 2 in abbreviation). Rat talin 2 function as the scaffolding ligand protein to bridge cell-cell adhesion and extracellular ligands, as well as transmembrane receptor proteins⁷⁰⁻⁷⁵. The talin 2 serves as a second control for integrin binding, cell-cell adhesion and productivity of effective conjugation⁷². Recent studies showed that talin-1 and talin-2 are important components involved in the regulation of integrin activation, mechanotransduction and cell-matrix adhesion stability⁷³. The talin rod binds to the cytoskeleton, whereas absence of talin is lethal^{71,75}. The talin 1 was up-regulated in the cervical cancer cells and identified as a direct target of microRNA miR-1285-3p⁷⁴. There are presently a few reports on the expression of *talin 2* gene in cancer cells^{70,71,73,75}. However, the genomes of a number of organisms encode a very well-conserved talin 2 protein in integrin regulation.

Slurp1: Secreted mammalian Ly6/urokinase plasminogen activator receptor-related protein-1 (Slurp1) is also known as ANUP, ARS, ArsB, LY6-MT, LY6LS and MDM. Rat Slurp1 is the protein expression of RGD1308768. The Slurp1 is a recently identified endogenous ligand of the alpha 7 subunit of nicotinic acetylcholine receptors ($\alpha 7$ nAChR or Chrna7)^{36,76-80}. The $\alpha 7$ nAChR is critical for the pathogenesis of *Escherichia coli*/K1 meningitis, a severe central nervous system infection of the neonates³⁶. Furthermore, $\alpha 7$ nAChR plays an essential anti-inflammatory role in immune homeostasis⁷⁷, whereas mammalian Slurp1 is a positive allosteric ligand for $\alpha 7$ nAChR that potentiates responses to ACh and elicits proapoptotic activity in keratinocytes. In contrast to nicotine, Slurp1 serves as a positive allosteric modulator to potentiate $\alpha 7$ nAChR

activity effectively as an endogenous $\alpha 7$ nAChR ligand^{76,80}. The Slurp1 is also the causative gene for a rare autosomal recessive skin disorder of transgressive palmoplantar keratoderma, Mal de Meleda^{79,80}. Mutations in *Slurp1* gene on patients were detected with Mal de Meleda^{79,80}. At present, little is known about the role of Slurp1 in the nervous system.

KB1: The keratin 1 (KB1) is also known as K1, CK1, EHK, EHK1, EPPK, KRT1, KRT1A and NEPPK. Keratin 1 is involved in many inflammatory diseases⁸¹⁻⁸⁵. Keratin 1 is a member of the G protein-coupled receptor 1 family participating in the NF- κ B signaling pathway and AGE-RAGE signaling pathway of diabetes complications. It was reported that keratin 1 silencing is able to make myocardial ischemia and reperfusion injury better by activating the Notch signaling pathway in mice⁸⁶. Keratin 1 gene silencing ameliorates myocardial ischemia-reperfusion injury via the activation of Notch signaling pathway in mouse models⁸⁶. It was revealed that hypoxic pulmonary artery remodeling was associated with a decrease in keratin 1⁸². Keratins 1 (K1) and keratin 10 (K10) are the primary keratins expressed in differentiated epidermis and mutations in K1/K10 are associated with human skin diseases^{83,85,87,88}. A mutation in a keratin 1 leading to a severe form of epidermal hyperkeratosis known as ichthyosis hystrix Curth-Macklin^{81,83}. Another novel causative mutation was identification in keratin 1 in diffuse palmoplantar keratoderma⁸⁸.

PGR: The progesterone receptor (PGR) is also known as NR3C3 and PR. In the signaling pathways, PGR is involved in the regulation of insulin signaling pathway and inhibits the PI3K-Akt signaling pathway⁸⁹⁻⁹⁷. Human PRG is located at 11q22-q23 as a member of the steroid receptor superfamily of nuclear receptors^{89,90,91}. The actions of progesterone are mediated by two functionally distinct isoforms (PR-A and PR-B) that are expressed from two alternative promoters from a single gene^{90,92-97}. Recent research revealed that progesterone and allopregnanolone are antinociceptive and attenuate orofacial pain and progesterone can exert protective effects through intracellular progesterone receptors (PGRs), membrane PGRs, sigma-1 receptors and its neuroactive metabolite allopregnanolone⁹⁸⁻¹⁰¹.

FRMD4A: The FERM domain containing 4A (FRMD4) is also known as CCAFCFA, FRMD4 and bA295P9.4. Rat FRMD4A is the protein expression of RGD1309050. As a member of the FERM superfamily, FRMD4A is involved in cell structure, transport

and signaling. The FRMD4A was recently identified to regulate epithelial polarity by connecting Arf6 activation with the PAR complex^{102,103}. It regulates cell polarity by playing an important role in the activation of ARF6, mediating the interaction between Par3 and the ARF6 guanine nucleotide exchange factor¹⁰³. Specifically, ARF6 is known to modulate nerve cell polarity and dendritic branching in hippocampal neurons and neurite outgrowth, while the FRMD4 domain is essential for determining cell polarity through interaction with Par3 during epithelial polarization^{102,103}. Particularly, FRMD4A was also regarded as a potential therapeutic target for the treatment of tongue squamous cell carcinoma in a molecular overexpression experiment¹⁰⁴. Tumor clinical stage and lymph node metastasis showed a statistically significant correlation with FRMD4A expression^{102,104,105} and FRMD4A upregulation in human squamous cell carcinoma promoted tumor growth and metastasis to be associated with poor prognosis¹⁰⁶. The FRMD4A also play a role in amyloidogenic and tau-related pathways in Alzheimer's disease (AD)¹⁰⁵. The FRMD4A-cytoskeleton signaling pathway was reported to modulate the cellular release of tau, which is also known as MAPT secreted from neurons in response to various stimuli and accumulates in the cerebrospinal fluid of AD patients¹⁰⁵. Polymorphisms of *FRMD4A* gene loci were usually suggested to be a key risk factor for AD¹⁰⁷⁻¹¹³. Several Genome-Wide Association Studies (GWASs) have led to the discovery of nine new loci of genetic susceptibility in AD^{107-109,111,112}. A genome-wide study identifies the *FRMD4A* gene on human Chr.10p13 as a risk locus for AD¹¹². In the brain transcriptomic and mechanistic elucidation of AD risk genes *in vivo* and *in vitro*, the expression of FRMD4A significantly decreased in the increasing AD-related neurofibrillary pathology¹¹⁰.

Additionally, DNA methylation in cord blood may mediate the effect of maternal smoking during pregnancy on birthweight of the offspring through FRMD4A and other genes^{108,109}. The homozygous mutation and DNA polymorphism of FRMD4A are frequently found associated with human DNA CpG methylation in prenatal tobacco smoke exposure or maternal pregnant smoking^{108,109,111}. Nicotine exposure was associated with a 2% increase in CpG methylation in FRMD4A ($p = 0.01$) and C1orf52 ($p = 0.001$) compared to no nicotine exposure¹⁰⁸. Large-scale genome-wide association study of Asian population reveals genetic factors in FRMD4A and other gene loci influencing smoking initiation and nicotine dependence¹⁰⁷. Therefore, FRMD4A has been listed as a key candidate gene of AD and other diseases with DNA methylation on smoking addition and nicotine dependence^{102,106-116}.

PPP2R2C: The gamma subunit of protein phosphatase 2 regulatory subunit B (PPP2R2C) is also known as B55 gamma, IMYPNO, IMYPNO1, PR52 and PR55G. Rat FRMD4A is known as Pr52. Research reports show that PPP2R2C encodes a tissue specific gam PPP2R2C was downregulated in glioma cells and human brain cancer patient samples^{117,118}. The PPP2R2C loss promoted castration-resistance and was associated with increased prostate cancer-specific mortality. The PPP2R2C was also a promising candidate gene of a temperament and character trait-based endophenotype of attention-deficit/hyperactivity disorder^{119,120}.

Rph3A: The rabphilin 3A (Rph3A) is a neuronal C2 domain tandem containing protein involved in vesicle trafficking¹²¹. Rabphilin-3A is also a cytosolic neuronal protein containing a C2-domain tandem that is involved in the neurotransmitter release cycle¹²¹. Rabphilin 3A are involved in synaptic vesicle transport and synaptic vesicle fusion and it has important biological roles in neurotransmitter release, hormone secretion and information transmission. Rabphilin-3A is expressed mainly in brain and it is a downstream target molecule of Rab3A small GTP-binding protein implicated in Ca²⁺-dependent neurotransmitter release¹²¹. Rabphilin-3A is a putative target protein for Rab3A small GTP-binding protein which is implicated in neurotransmitter release. Recent research indicated that rabphilin 3A was a novel target for the treatment of levodopa-induced dyskinesias¹²² and it was a targeted autoantigen in lymphocytic infundibulo-neurohypophysitis too¹²³. Decreased rabphilin 3A immunoreactivity in Alzheimer's disease was associated with A β burden¹²⁴. The anti-rabphilin-3A antibody (Rab3A-Ab) positivity was frequently reported¹²⁵. However, there is little information on the potential therapeutic target of rabphilin 3A in tobacco smoke and relevant diseases.

HtrA3: High-temperature requirement serine protease A3 (HtrA3), belongs to a family of unique cellular homooligomeric and ATP-independent serine proteases¹²⁶⁻¹³⁹. Rat HtrA3 is the protein expression of RGD1308120. Recently, HtrA3 was identified as a potential diagnostic marker for early detection of preeclampsia disease, lung cancer, ovarian cancer, endometrial cancer and other disorders^{24-27,126-141}. It is regarded as a tumor suppressor and a potential therapeutic target in different cancers^{25,26,127,132,134-138}. In addition, HtrA3 also plays crucial roles in placental development and the pregnant smoking-related diseases^{131,133,139,140}. The HtrA3 is reduced in ovarian cancers regardless of stages¹³⁶, while its protein expression is closely associated with changing in oxygen tension in the placenta^{137,140}.

Functional enrichment analyses of GO terms and KEGG pathways: To understand the potential effects of exposure to nicotine on biological function, the 382 shared DEGs were adopted to conducted enrichment analyses of GO terms and KEGG pathways.

Enrichment analysis of GO terms: The clusterProfiler software⁴⁸ was adopted for functional enrichment analysis of the 382 shared DEGs. A total of 4717 entries of GO categories were initially enriched from the 382 shared DEGs, whereas there were 28 significant GO categories with the adjust p<0.05 (Fig. 3a, Table 2).

Among these significant GO categories, 23 (82.14%) were enriched in many interesting biological processes (Fig. 3a, Table 2). These enriched biological processes included cellular response to metal ion (13 entries), neuronal death (18 entries), response to zinc ion (7 entries), response to osmotic stress (9 entries), myeloid cell differentiation (17 entries), response to glucocorticoid (14 entries), regulation of neuronal death (16 entries), neuron apoptotic process (14 entries), regulation of neuronal synaptic plasticity (7 entries), regulation of neuron apoptotic process (13 entries), cellular response to inorganic substance (14 entries), osteoclast differentiation (8 entries), response to corticosteroid (14 entries), regulation of long-term neuronal synaptic plasticity (5 entries), negative regulation of neuron death (12 entries), negative regulation of neuron apoptotic process (10 entries), response to ischemia (6 entries), response to alkaloid (10 entries), monocarboxylic acid biosynthetic process (10 entries), regulation of protein serine/threonine kinase activity (17 entries), response to purine-containing compound (11 entries), myeloid leukocyte differentiation (11 entries) and ribonucleotide metabolic process (14 entries). It was found that 17.86% (5 entries) of the significantly enriched entries were the cell composition categories of GO terms, including peroxisomal membranes, microbody membrane, clathrin-coated pit, dendritic spine and neuron spine (Fig. 3a, Table 2). However, there were no findings of significantly enriched molecular function entries of GO terms.

Enrichment analysis of GO terms showed the following interesting biological processes, i.e. cellular response to metal ion, neuron death, response to zinc ion, response to osmotic stress, myeloid cell differentiation, response to glucocorticoid, regulation of neuron death, neuron apoptotic process, regulation of neuronal synaptic plasticity, regulation of neuron apoptotic process, cellular response to inorganic substance, osteoclast differentiation, response to corticosteroid, regulation of long-term neuronal synaptic plasticity, negative regulation of neuron death, negative regulation of neuron apoptotic process, response to ischemia, response to alkaloid,

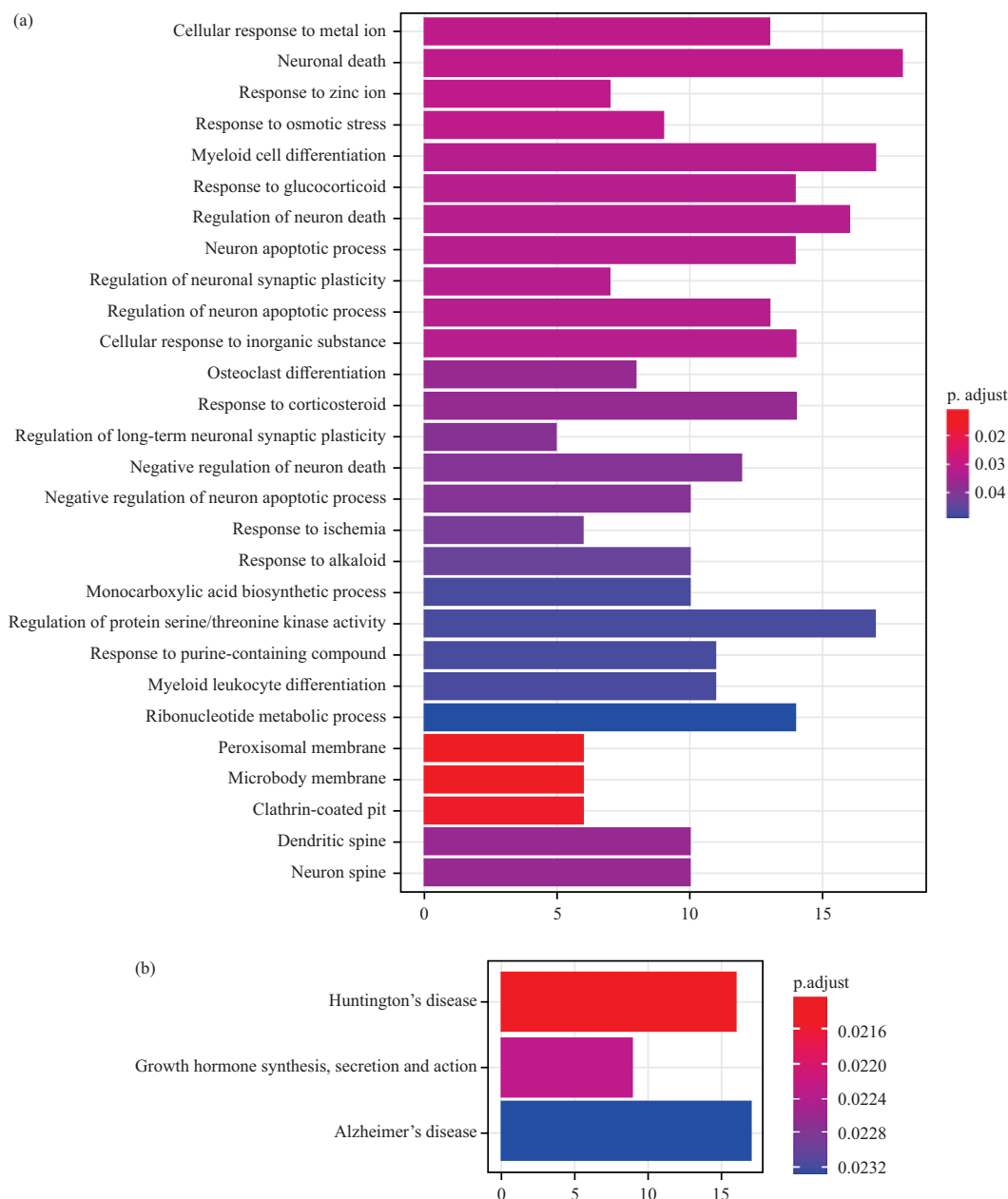


Fig. 3(a-b): Functional enrichment analysis of (a) GO terms and (b) KEGG pathways

monocarboxylic acid biosynthetic process, regulation of protein serine/threonine kinase activity, response to purine-containing compound, myeloid leukocyte differentiation and ribonucleotide metabolic process. These are potential physiological impacting biological processes after smoking or tobacco intake in nicotine addiction. Among these GO entries enriched, seven were related to neuronal development, death and apoptosis, in which Hif1a, Apoe, Prdx2, Hyou1, Map2k4, Bax, Kras, Bcl2l1, Faim2 and Pcp4 were the most significant DEGs. In terms of GO categories of cell

composition, there were significantly enriched entries of peroxisomal membrane, microbody membrane, clathrin-coated pit, dendritic spine and neuron spine. It indicated that these categories of cell composition would be affected in smoking or nicotine addiction or tobacco dependence.

Enrichment analysis of KEGG pathways: A total of 260 KEGG pathways were enriched and retrieved from the KEGG pathway database by entering the 382 shared DEGs⁴⁸. After statistical analysis of enrichment, there were three significantly

Table 2: Enrichment analysis of GO terms

GO ID	Gene ontology	Description	Gene ratio	Adjusted p-values
GO:0071248	BP	Cellular response to metal ion	13/243	0.0303
GO:0070997	BP	Neuron death	18/243	0.0303
GO:0010043	BP	Response to zinc ion	7/243	0.0303
GO:0006970	BP	Response to osmotic stress	9/243	0.0303
GO:0030099	BP	Myeloid cell differentiation	17/243	0.0320
GO:0051384	BP	Response to glucocorticoid	14/243	0.0320
GO:1901214	BP	Regulation of neuron death	16/243	0.0320
GO:0051402	BP	Neuron apoptotic process	14/243	0.0320
GO:0048168	BP	Regulation of neuronal synaptic plasticity	7/243	0.0320
GO:0043523	BP	Regulation of neuron apoptotic process	13/243	0.0320
GO:0071241	BP	Cellular response to inorganic substance	14/243	0.0320
GO:0030316	BP	Osteoclast differentiation	8/243	0.0365
GO:0031960	BP	Response to corticosteroid	14/243	0.0366
GO:0048169	BP	Regulation of long-term neuronal synaptic plasticity	5/243	0.0390
GO:1901215	BP	Negative regulation of neuron death	12/243	0.0390
GO:0043524	BP	Negative regulation of neuron apoptotic process	10/243	0.0390
GO:0002931	BP	Response to ischemia	6/243	0.0413
GO:0043279	BP	Response to alkaloid	10/243	0.0433
GO:0072330	BP	Monocarboxylic acid biosynthetic process	10/243	0.0466
GO:0071900	BP	Regulation of protein serine/threonine kinase activity	17/243	0.0466
GO:0014074	BP	Response to purine-containing compound	11/243	0.0468
GO:0002573	BP	Myeloid leukocyte differentiation	11/243	0.0468
GO:0009259	BP	Ribonucleotide metabolic process	14/243	0.0489
GO:0005778	CC	Peroxisomal membrane	6/242	0.0105
GO:0031903	CC	Microbody membrane	6/242	0.0105
GO:0005905	CC	Clathrin-coated pit	6/242	0.0161
GO:0043197	CC	Dendritic spine	10/242	0.0362
GO:0044309	CC	Neuron spine	10/242	0.0362

enriched KEGG pathways with adjust $p < 0.05$ (Fig. 3b, Table 3). Those significantly enriched KEGG pathways were Huntington's disease (rno05016, adjust $p = 0.0212$), growth hormone synthesis, secretion and action (rno04935, adjust $p = 0.0223$) and Alzheimer's disease (rno05010, adjust $p = 0.0233$). In addition, in the enriched KEGG pathways, there were also many disease-related entries and energy metabolism relevant pathways with nearly significant adjusted p-values (Table 3). In these resulted in disease-related signaling pathways, eleven potential neuropathic disease-related genes were explored in the PPI network, including Psma3, Psmb2, Psmd13, Psmc5, Plcb2, Ppif, Tubb2b, Ndufs6, Ndufs7, Ndubf8 and Ndubf11. In addition, there are a number of nicotine- and disease-related pathways that enriched with insignificant p adjusted values, including Prion disease (rno05020), Parkinson disease (rno05012), pathways of neurodegeneration-multiple diseases (rno05022), circadian rhythm (rno04710), long-term depression (rno04730), autophagy-animal (rno04140), p53 signaling pathway (rno04115), TNF signaling pathway (rno04668), HIF-1 signaling pathway (rno04066), neurotrophin signaling pathway (rno04722) and nicotine addiction (rno05033).

Enrichment analysis of KEGG pathways showed that there were three significant enriched pathways for the 382 shared

DEGs, with the adjusted $p < 0.05$. These interesting KEGG pathways were Huntington's disease (rno05016), growth hormone synthesis and secretion and action (rno04935) and Alzheimer's disease (rno05010).

Huntington's disease: The Huntington's disease (rno05016), also known as Huntington's chorea or the dementia in Huntington's disease. It is an autosomal dominant neurodegenerative disorder¹⁴². It is caused by a mutation in the Huntington gene on chromosome 4, which produces the mutant protein and accumulates in the brain^{142,143}. In the study, 16 genes were enriched in the Huntington's disease pathway, which were Psmb2 (Proteasome Subunit Beta Type-2), Psma3 (Proteasome Subunit Alpha Type-3), Psmd13 (26S Proteasome Non-ATPase Regulatory Subunit 13), Psmc5 (26S Proteasome Regulatory Subunit 8), Ndufs6, Tubb2b, Ndubf8, Ap2m1, Ppif, Gria1 (glutamate receptor 1), Creb1, Ndubf11, Ndufs7, Bax, Plcb2 and Grm5.

Growth hormone synthesis, secretion and action: The Growth Hormone (GH) production is governed by multiple neuroendocrine factors from the hypothalamus and other regulators from the pituitary and peripheral organs. Growth hormone synthesis, secretion and action (rno04935) is

Table 3: Enrichment analysis of KEGG pathways

KEGG ID	Description	Gene ratio	p-value	Adjusted p-value
rno05016	Huntington's disease	16/156	0.0001	0.0212
rno04935	Growth hormone synthesis, secretion and action	9/156	0.0002	0.0223
rno05010	Alzheimer's disease	17/156	0.0003	0.0233
rno05020	Prion disease	13/156	0.0010	0.0520
rno05022	Pathways of neurodegeneration-multiple diseases	18/156	0.0014	0.0520
rno04932	Non-alcoholic fatty liver disease	9/156	0.0014	0.0520
rno05012	Parkinson disease	12/156	0.0015	0.0520
rno04728	Dopaminergic synapse	8/156	0.0018	0.0532
rno05166	Human T-cell leukemia virus 1 infection	11/156	0.0041	0.0926
rno04723	Retrograde endocannabinoid signaling	8/156	0.0043	0.0926
rno04919	Thyroid hormone signaling pathway	7/156	0.0046	0.0926
rno04152	AMPK signaling pathway	7/156	0.0060	0.1035
rno04910	Insulin signaling pathway	7/156	0.0095	0.1320
rno04024	cAMP signaling pathway	9/156	0.0102	0.1320
rno05017	Spinocerebellar ataxia	7/156	0.0117	0.1326
rno05224	Breast cancer	7/156	0.0126	0.1326
rno05014	Amyotrophic lateral sclerosis	13/156	0.0128	0.1326
rno05162	Measles	7/156	0.0134	0.1344
rno05161	Hepatitis B	7/156	0.0196	0.1456
rno04714	Thermogenesis	9/156	0.0213	0.1500
rno05165	Human papillomavirus infection	11/156	0.0402	0.2043
rno04080	Neuroactive ligand-receptor interaction	11/156	0.0409	0.2043
rno05202	Transcriptional misregulation in cancer	7/156	0.0559	0.2596
rno05167	Kaposi sarcoma-associated herpesvirus infection	7/156	0.0810	0.3190
rno04151	PI3K-Akt signaling pathway	9/156	0.1372	0.3751
rno05163	Human cytomegalovirus infection	7/156	0.1377	0.3751
rno05132	Salmonella infection	7/156	0.1558	0.3932
rno04060	Cytokine-cytokine receptor interaction	7/156	0.1750	0.4138

essential for understanding the endocrine regulation network of animal growth hormone synthesis and secretion. In particular, Creb1, Kras, Gsk3b, Plcb2, Sst, Junb, Map2k4, Fos and Sstr2 were enriched in this pathway too. Growth hormone synthesis, secretion and action involves several other interesting pathways in the same study, such as the cAMP signaling pathway (rno04024, adjusted p = 0.1320), calcium signaling pathway (rno04020), MAPK signaling pathway (rno04010) and JAK-STAT signaling pathway (rno04630). These pathways are important for cell growth and metabolism at the cellular and intracellular levels.

Alzheimer's disease: The Alzheimer's disease (AD, rno05010) is a degenerative disease of the central nervous system with complex pathogenesis and clinical manifestations in progressive cognition, memory-related impairment and executive dysfunction. There were 17 DEGs enriched in the Alzheimer's disease pathway (rno05010), in which 12 genes also appeared in the Huntington's disease pathway (rno05016). These 12 genes are Ndufs6, Psmb2, Tubb2b, Psma3, Nsufb8, Psmc5, Ppif, Ndubf11, Ndufs7, Psmd13, Plcb2 and Grm5. The rest of the genes are Gapdh, Apoe, Kras and Gsk3b. The Alzheimer's disease pathway overlaps and is associated with the pathways of oxidative phosphorylation, calcium signaling pathway, Wnt signaling pathway, AGE-RAGE

signaling pathway in diabetic complications, impaired neuronal insulin signaling protein processing in endoplasmic reticulum. These pathways interact with each other and lead to the processes of cell growth and metabolism and apoptosis.

In addition, there are a number of nicotine- and disease-related pathways that enriched with insignificant p adjusted values, including prion disease (rno05020), Parkinson's disease (rno05012), pathways of neurodegeneration-multiple diseases (rno05022), circadian rhythm (rno04710), long-term depression (rno04730), autophagy-animal (rno04140), p53 signaling pathway (rno04115), TNF signaling pathway (rno04668), HIF-1 signaling pathway (rno04066), neurotrophin signaling pathway (rno04722) and nicotine addiction (rno05033). For instance, there were 18 DEGs enriched in the pathways of neurodegeneration-multiple diseases that are associated with a variety of neurodegenerative diseases like Alzheimer's disease, Huntington's disease, Parkinson's disease, myasthenia gravis and spinocerebellar ataxia. There were 13 DEGs enriched in the pathway of prion disease which is caused by accumulation of the Pathological Prion Protein (PrPSc) in the central nervous system¹⁴⁴. There were 12 DEGs enriched in the pathway of Parkinson's disease (PD) which is a chronic neurodegenerative disease endangering the health of the middle-aged and elderly. The pathological feature of Parkinson's disease is the progressive loss of dopaminergic

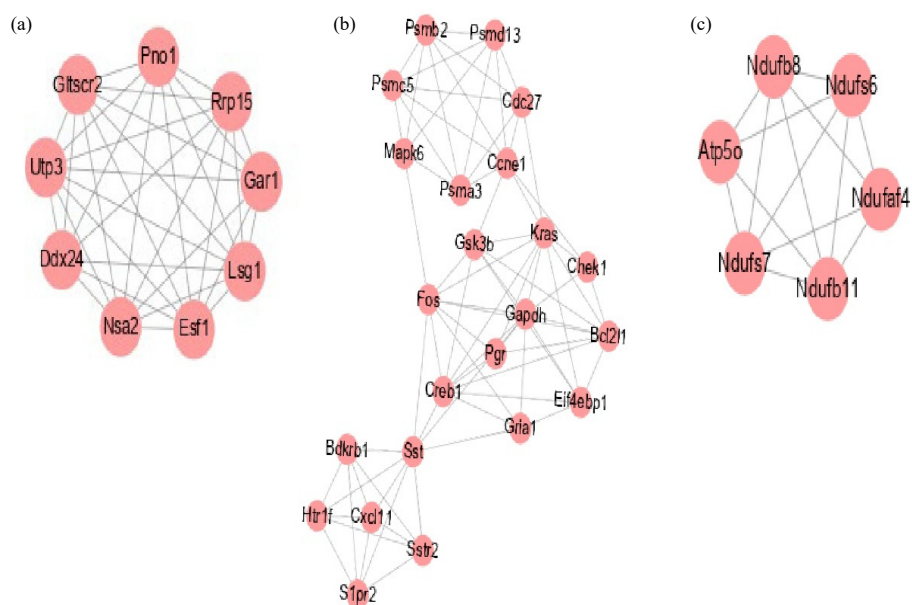


Fig. 4(a-c): Three key modules identified with the MMC algorithm of CytoHubba in the PPI network of 382 DEGs, (a) Cluster 1 (score 8.75), (b) Cluster 2 (score 6.818) and (c) Cluster 3 (score 5.6)

neurons in the substantia nigra of the brain, resulting in the lack of dopamine content in the striatum leading to dyskinesia¹⁴⁵. The pathway of nicotine addiction was also enriched due to exposure to nicotine in the study. Besides Alzheimer's disease and Huntington's disease, many reports revealed that nicotine addiction is also associated with Parkinson's disease^{145,146}, lung diseases^{147,148}, cardiovascular disease¹⁴⁸⁻¹⁵⁰ and gastrointestinal disease^{148,149,151} in exposure to nicotine.

In brief, the effects of exposure to nicotine are mainly involved in synaptic plasticity, the cellular and intracellular processes of the nervous system's cell growth and development and apoptosis, tendency prone to low immunity and inflammation and nerve signal transduction phenomena, as well as disorders. Ultimately, it will affect the level of attention and cognitive and give rise to the incidence of neurological diseases like Alzheimer's disease and Parkinson's disease.

Analysis of PPI network and identification of key modules and hub genes: The PPI network and 126 nodes of 382 DEGs generated on the STRING website were retrieved and constructed by the StringApp software in Cytoscape⁵². This PPI network was visualized, analyzed and displayed by the Cytoscape software MCODE⁵³ and CytoHubba⁵⁴ and the other Cytoscape plugins⁵².

Three densely connected subnetworks were identified as the key modules. The PPI network totally covered 284 points

and 585 lines with those three key modules or subnetworks (Fig. 4). Module 1 (Fig. 4a) contained 9 genes and 35 lines, with an assessment score of 8.750. Module 1 was involved in rRNA processing, ribosome synthesis, ribonucleoprotein complex, cellular components, ncRNA processing, 5.8S rRNA maturation, rRNA metabolism, rRNA and RNA processing and ncRNA metabolic processes, etc. Module 2 (Fig. 4b) included 23 points and 75 lines, with an assessment score of 6.818. Module 2 was involved in proteasomal protein decomposition process protease body catabolism process, etc. Module 3 covered 6 points and 14 lines (Fig. 4c), with an assessment score of 5.6. Module 3 was involved in the ATP synthase and NADH dehydrogenase (ubiquinone) related processes.

The subnetworks of modules were further explored by the BINGO software⁵⁵ with the interested DEGs.

Next, ten hub genes were identified with the MMC algorithm of CystoHubba¹⁵² in Fig. 5. The 10 hub genes were identified as GAPDH, Gsk3b, Gria1, Creb1, Fos, Polr3a, Bcl2l1, Kat2b, Kras and Kdm6a, respectively (Fig. 5). Analysis with the BINGO software⁵⁵ showed that these 10 hub genes were mainly related to the following biological processes (Fig. 5), including ribosome biogenesis, ribonucleoprotein complex biogenesis, rRNA processing and rRNA metabolic process, ncRNA processing and ncRNA metabolic process, cellular component biogenesis, RNA processing and RNA metabolic process, nucleic acid metabolic process, cellular nitrogen compound metabolic process and nitrogen compound metabolic process, etc.

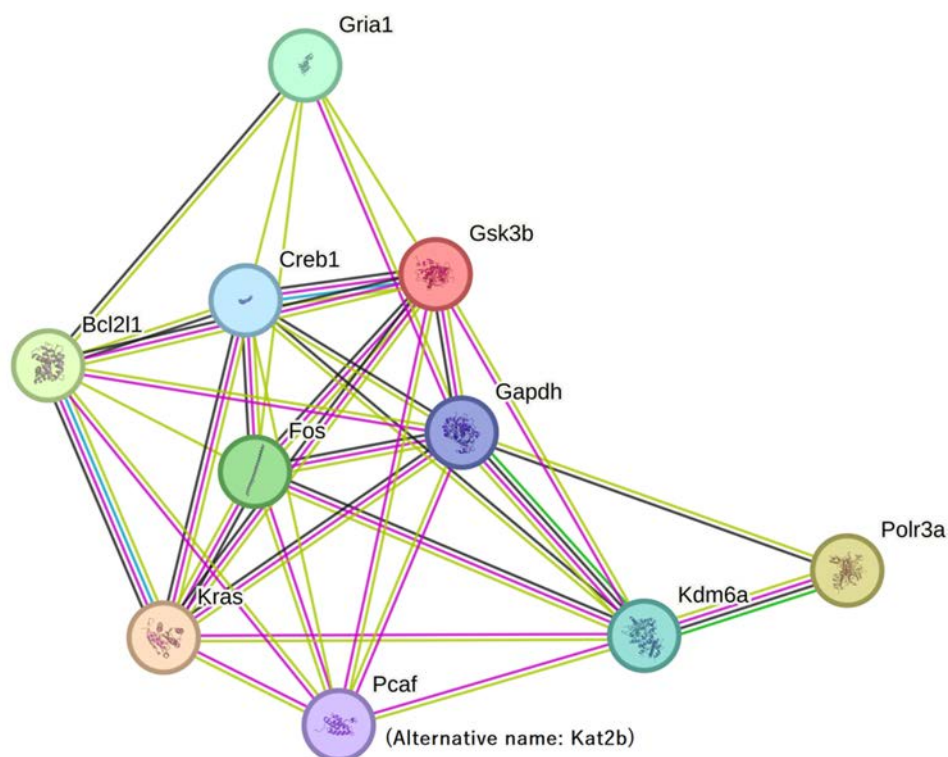


Fig. 5: Network diagram of the 10 identified hub genes

The PPI network analysis identified three densely connected subnetworks as the key modules (Fig. 5). It involved in the processing and metabolic processes of rRNA and ncRNA, the process of proteasomal protein decomposition and proteases body catabolism and the ATP synthase and NADH dehydrogenase (ubiquinone) related processes, respectively. It also identified and defined 10 hub genes from the key modules that were shown in both the PPI network and subnetworks.

The network diagram shows the 10 identified hub genes (GAPDH, Gsk3b, Gria1, Creb1, Fos, Polr3a, Bcl2l1, Kat2b, Kras and Kdm6a) generated in the STRING website (Fig. 5). These hub genes played important roles in the process of smoking and exposure to nicotine as well as nicotine addiction or tobacco dependence in recent studies^{28,37,153-165}. However, there are presently only a few reports on these subnetworks/modules and hub genes identified in the study. Further research will be needed to elucidate the molecular mechanism of these subnetworks/modules and hub genes in the progression of the process of smoking and exposure to nicotine as well as nicotine addiction or tobacco dependence.

The study explored the possible effects of exposure to nicotine on the gene expression profiles of rat brain tissues

based on the microarray dataset GSE59895 in the GEO database. Many DEGs were systematically analyzed in the rat brain tissues after environmental exposure to nicotine. However, there is a non-negligible limitation of animal treatments, i.e., the rats were dealt without toxicant inhalation or ingestion in the experiment. In animal experiment¹, the toxicants were profiled in up to 8 different rat tissues by obtaining tissue samples from test compound-treated and vehicle control-treated rats in biological triplicates for gene expression analysis. Whether the toxicant reagents were effective, the experiments were not carried out in an ideal experimental condition with the same cases of human smoking and/or second-hand smoking. This should be solved in subsequent experiments.

CONCLUSION

In the present study, the effects of exposure to nicotine were evaluated based on the gene expression profiles of rat brain tissues with the microarray dataset GSE59895. In total, 382 shared DEGs were identified and explored within the three case groups of exposure to nicotine. It was found that exposure to nicotine would result in some disease-related DEGs. These DEGs could increase the environmental risks of

Huntington's disease, Alzheimer's disease, Parkinson's disease and other multiple neurodegeneration diseases. In the disease-related signaling pathways, eleven potential neuropathic disease-related genes, i.e., Psma3, Psmb2, Psmd13, Psmc5, Plcb2, Ppif, Tubb2b, Ndufs6, Ndufs7, Ndubf8 and Ndubf11, may complement the treatment of neurodegenerative diseases. These eleven potential neuropathic disease-related genes may complement the treatment of neurodegenerative diseases. The study provided new insights into the effects of environmental exposure to nicotine on the gene expression profiles in rat brain tissues. It may serve as a research foundation for further attempt at exploring the molecular mechanism of diseases related to exposure to nicotine.

SIGNIFICANCE STATEMENT

Nicotine relevant smoking causes many serious issues of environmental pollution and complicated harm to human health. The present study aimed to evaluate the effects of exposure to nicotine on the differentially expressed genes (DEGs) of rat brain tissues based on the microarray dataset. There were a totally of 382 shared DEGs between different case groups in the experiment, whereas 9 common shared DEGs were found among all three groups. The significant enrichments of 28 GO terms and 3 KEGG pathways were comprehensively analyzed with their corresponding functionally enriched genes. The study further identified and explored 3 key modules and 10 hub genes in the resulting protein-protein interaction network, while eleven potential neuropathic disease-related genes were found.

ACKNOWLEDGMENTS

The authors are grateful to research group of Dr. Auerbach S. for the generous publication of microarray dataset GSE59895 in GEO database. The authors thank the referees for their constructive comments. It is jointly funded by the Project of Anhui Provincial Educational Commission on Nature Science Foundation (No. KJ2019ZD36), the National and Provincial Projects of College Student Innovation and Entrepreneurship Training Program (No. 202210371025, No. 202310371131) and the Biological and Medical Sciences of Applied Summit Nurturing Disciplines in Anhui Province (Anhui Provincial Education Secretary Department [2023]13).

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