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Screening Key Pathogenic Genes and Small Molecule Compounds for PNET

Qi Zhou, MD, PhD, Hao Liu, MD,† Junsi Liu, MD,‡ Zhendong Liu, MD, PhD,§ Caixia Xu, MD,‡ Haiyu Zhang, MD, PhD,‖ and Chen Xin, MD, PhD‡*

Summary: Primitive neuroectodermal tumors (PNET) are rare malignant tumors, but the mortality rate of the patients is extremely high. The aim of this study was to identify the hub genes and pathways involved in the pathogenesis of PNET and to screen the potential small molecule drugs for PNET. We extracted gene expression profiles from the Gene Expression Omnibus database and identified differentially expressed genes (DEGs) through Limma package in R. Two expression profiles (GSE14295 and GSE74195) were downloaded, including 33 and 5 cases separately. Four hundred sixty-eight DEGs (161 upregulated; 307 downregulated) were identified. Functional annotation and KEGG pathway enrichment of the DEGs were performed using DAVID and Kobas. Gene Ontology analysis showed the significantly enriched Gene Ontology terms included but not limited to mitosis, nuclear division, cytoskeleton, synaptic vesicle, syntaxin binding, and GABA A receptor activity. Cancer-related signaling pathways, such as DNA replication, cell cycle, and synaptic vesicle cycle, were found to be associated with these genes. Subsequently, the STRING database and Cytoscape were utilized to construct a protein-protein interaction and screen the hub genes, and we identified 5 hub genes (including CCNB1, CDC20, KIF11, KIF2C, and MAD2L1) as the key biomarkers for PNET. Finally, we identified potential small molecule drugs through CMap. Seven small molecule compounds, including trichostatin A, luteolin, repaglinide, clomipramine, lorglumide, vorinostat, and resveratrol may become potential candidates for PNET drugs.

Key Words: PNET, bioinformatics, differentially expressed genes, small molecule compounds

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Primitive neuroectodermal tumor (PNET) is a rare and highly malignant small round cell tumor.[1] Developing from primitive nerve cells of the nervous system, this disease affects both males and females of all ages and mainly occurs in adolescents and children. Accompanied by the low incidence rate, it features high-lever of malignancy, rapid progress, high recurrence and metastasis rate, and poor prognosis.[2] To date, few effective approaches are available to improve the prognosis of patients.[3] Therefore, a comprehensive analysis is needed to discover biomarkers of the disease and small molecule drugs that can improve the patients' treatment strategy.

After decades of hard work, researchers have discovered biomarkers that have predictive effect for the prognosis of PNET. Xiao et al[4] reported ZBTB16 effectively distinguished PNET from other small round blue cell tumor mimics. Choi et al[5] found that increased expression of Rad51 in sPNET patients was significantly correlated with decreased survival rate, which supports the hypothesis that Rad51 may enhance radiation resistance. Picard et al identified LIN28 and OLIG2 were potential markers for the survival and metastasis of primitive neuroectodermal brain tumors in the childhood central nervous system.[6] However, although there have been so many biomarkers used in the diagnosis and treatment of PNET, the treatment outcome remains unsatisfactory. The main reason for this dilemma is that the pathological process of the tumor is extremely complex, and there are even different molecular subtypes, so the prognosis of PNET patients cannot be determined by a single factor. Therefore, exploring the abnormal expression level of genes in the pathological development of PNET through sequencing or gene chip technology is particularly important for understanding the development mechanism of the disease and finding therapeutic targets.

At present, gene chip technology has been widely used in the diagnosis and treatment of diseases. This technology can quickly and accurately analyze high-level information, and it provides the important theoretical basis for sequence analysis, gene expression, and genome research.[7] In recent years, various public free chip databases can be used by researchers on the Internet for in-depth research. However, single PNET chip analysis may result in statistical deviation, and then lead to results that are not accurate enough. Therefore, integrating PNET chip data to explore specific and sensitive biomarkers will be more meaningful for clinical practice.

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From the *Scientific Research Management Office; ‡Department of Neurosurgical laboratory; ‖Department of Cardiology, The First Affiliated Hospital of Harbin Medical University, Harbin Heilongjiang Province; †The second Hospital of Harbin, Harbin, Heilongjiang Proviance; and §Department of Orthopaedics, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, School of Clinical Medicine, Henan University, Zhengzhou, Henan, China.

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Q.Z., H.L., J.L., and Z.L. contributed equally.

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Reprints: Chen Xin, MD, PhD, The First Affiliated Hospital of Harbin Medical University, 23 Youzheng St, Harbin 150001, Heilongjiang Province, China (e-mail: chenxin_tracy@yeah.net).

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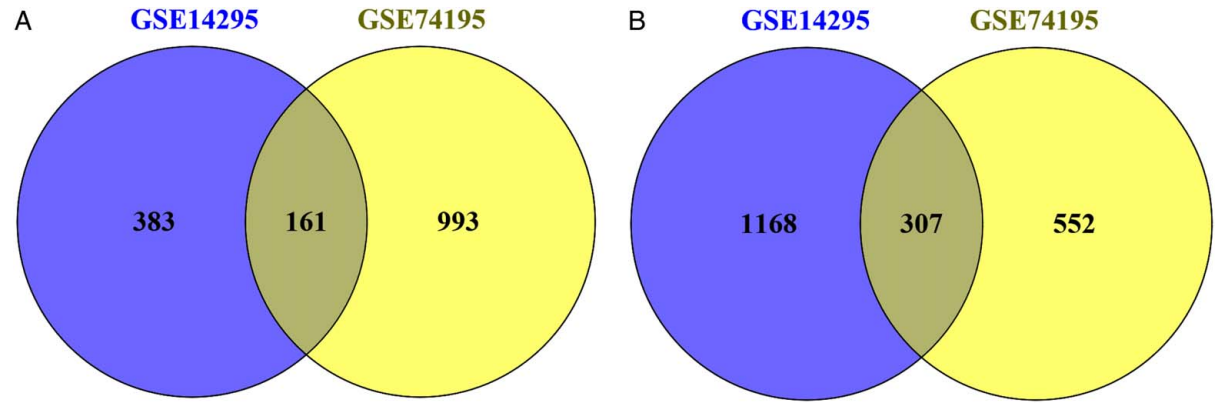

FIGURE 1. The Venn diagrams of DEGs in 2 different PNET microarray datasets. (A) upregulated DEGs; (B) downregulated DEGs.

On these grounds, in this study, we first downloaded 2 PNET microarray datasets—GSE14295 and GSE74195 from GEO (Gene Expression Omnibus) and further obtained common differentially expressed genes. Then, through a series of bioinformatic analyses, we found important genes playing key roles in this disease. Even we skillfully screened out some small molecule compounds to correct these abnormally expressed causative genes to achieve the potential of treating PNET. Thus, this study was particularly innovative and of great clinical significance and offered new perspectives for elucidation of PNET through scientific and reliable approaches.

TABLE 1. Top 10 Meaningful Terms of 3 GO Functions From DAVID Analysis

Term	Description	Count	*P*
CC			
GO:0045202	Synapse	50	1.24E-19
GO:0044456	Synapse part	41	9.57E-19
GO:0043005	Neuron projection	39	2.50E-12
GO:0030424	Axon	25	5.84E-11
GO:0008021	Synaptic vesicle	18	6.75E-11
GO:0030136	Clathrin-coated vesicle	20	1.35E-08
GO:0030135	Coated vesicle	21	5.77E-08
GO:0019717	Synaptosome	15	2.04E-07
GO:0005819	Spindle	19	4.02E-07
GO:0005856	Cytoskeleton	74	8.14E-07
MF			
GO:0000149	SNARE binding	9	5.13E-06
GO:0019905	Syntaxin binding	8	1.49E-05
GO:0003774	Motor activity	15	3.02E-05
GO:0016917	Motor activity	7	3.33E-05
GO:0003777	Motor activity	11	4.02E-05
GO:0005200	Structural constituent of cytoskeleton	9	8.39E-04
GO:0017075	Syntaxin-1 binding	4	9.92E-04
GO:0004890	GABA A receptor activity	5	0.001475309
GO:0015631	Tubulin binding	10	0.001524052
GO:0008017	Microtubule binding	8	0.003244914
BP			
GO:0007067	Mitosis	30	3.27E-12
GO:0000280	Nuclear division	30	3.27E-12
GO:0000087	M phase of mitotic cell cycle	30	5.16E-12
GO:0048285	Organelle fission	30	9.00E-12
GO:0000279	M phase	36	9.76E-12
GO:0051301	Cell division	34	1.00E-11
GO:0022403	Cell cycle phase	40	2.60E-11
GO:0000278	Mitotic cell cycle	37	6.40E-11
GO:0007268	Synaptic transmission	32	2.77E-10
GO:0007017	Microtubule-based process	29	5.07E-10

DAVID indicates the database for annotation, visualization and integrated discovery; GO, gene ontology.

MATERIALS AND METHODS

Microarray Data Information

The gene expression profiles of GSE14295 and GSE74195 were downloaded from the GEO database. Based on the GPL6102 Platforms (Illumina human-6 v2.0 expression beadchip), the GSE14295 data set contained 33 PNET samples and 14 normal brain samples.[8] Based on the GPL570 Platform (Affymetrix Human Genome U133 Plus 2.0 Array), the GSE74195 data set contained 5 PNET samples and 5 normal brain tissue samples.[9]

Identification of DEGs in PNET

We identified DEGs based on the series matrix file using Limma package in R software (version 3.5.0). The cutoff standard for DEGs was set as $|\log FC| > 1$ and $P < 0.05$. The preliminary candidate genes were respectively divided into upregulated DEGs and downregulated DEGs for subsequent analysis. The pheatmap package in R was used to generate the heatmap, and the GSE74195 data set was used as the reference. One hundred sixty-one upregulated DEGs and 307 downregulated DEGs were visualized in the heatmap.

Gene Ontology Functional Enrichment and KEGG Pathway Analysis of DEGs

GO (Gene Ontology), a database established by the Gene Ontology Consortium, aims to establish an updatable semantic-lexical standard that is applicable to various species and can limit and describe the functions of genes and proteins. KEGG (Kyoto Encyclopedia of Genes and Genomes) is a well-known bioinformatic analysis database that can retrieve the signal pathways involved in the target genes to reveal the functional mechanism of the genes. The DAVID online database and Kobas online tool were used to perform Gene Ontology (GO)-based functional enrichment and KEGG pathway enrichment analyses.[10] The cutoff value was defined as $P < 0.05$.

Construction of the PPI Network

PPI network (protein-protein interaction network) analysis contributes to the investigation of the molecular

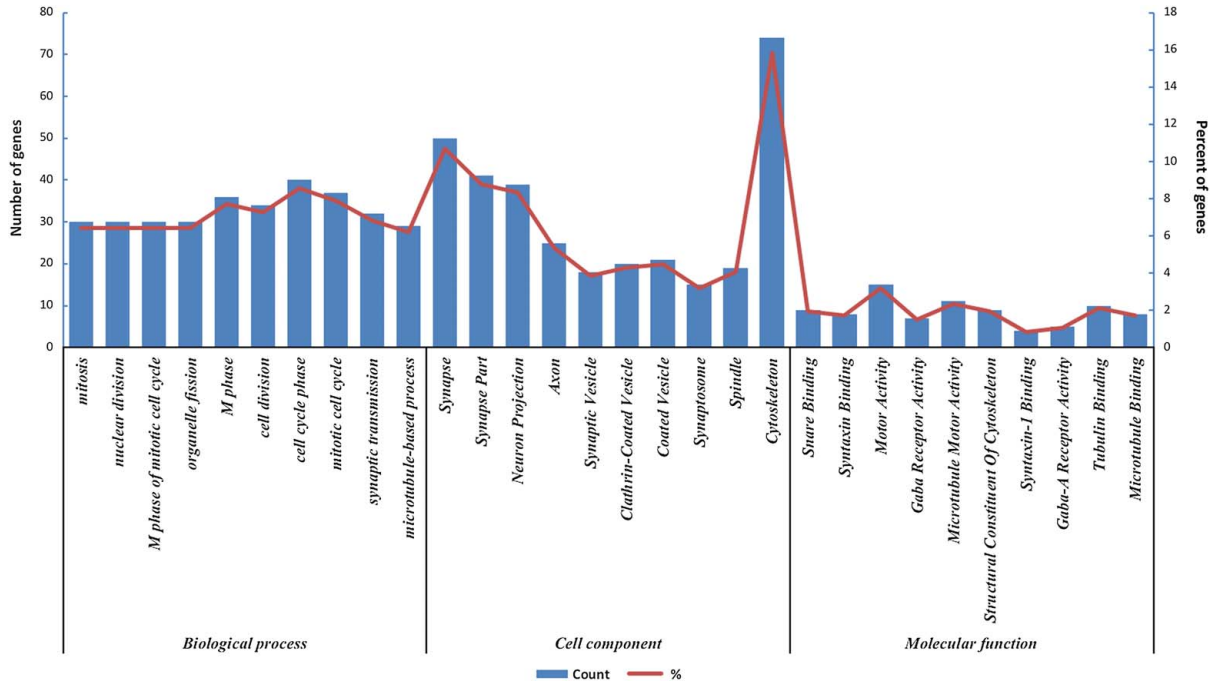

FIGURE 2. GO analysis of DEGs in PNET from 3 category (biological process, cell component, molecular functions).

mechanism of diseases and the discovery of new drug targets from a systematic perspective. The STRING database (http://string-db.org/) can score and integrate known and predicted associations to form a comprehensive protein network, which covers >1100 organisms.[11] Based on the STRING database, DEGs were used to construct a PPI network with an Interaction score of 0.9. Visualization of the PPI network and screening for hub genes by scrutinizing

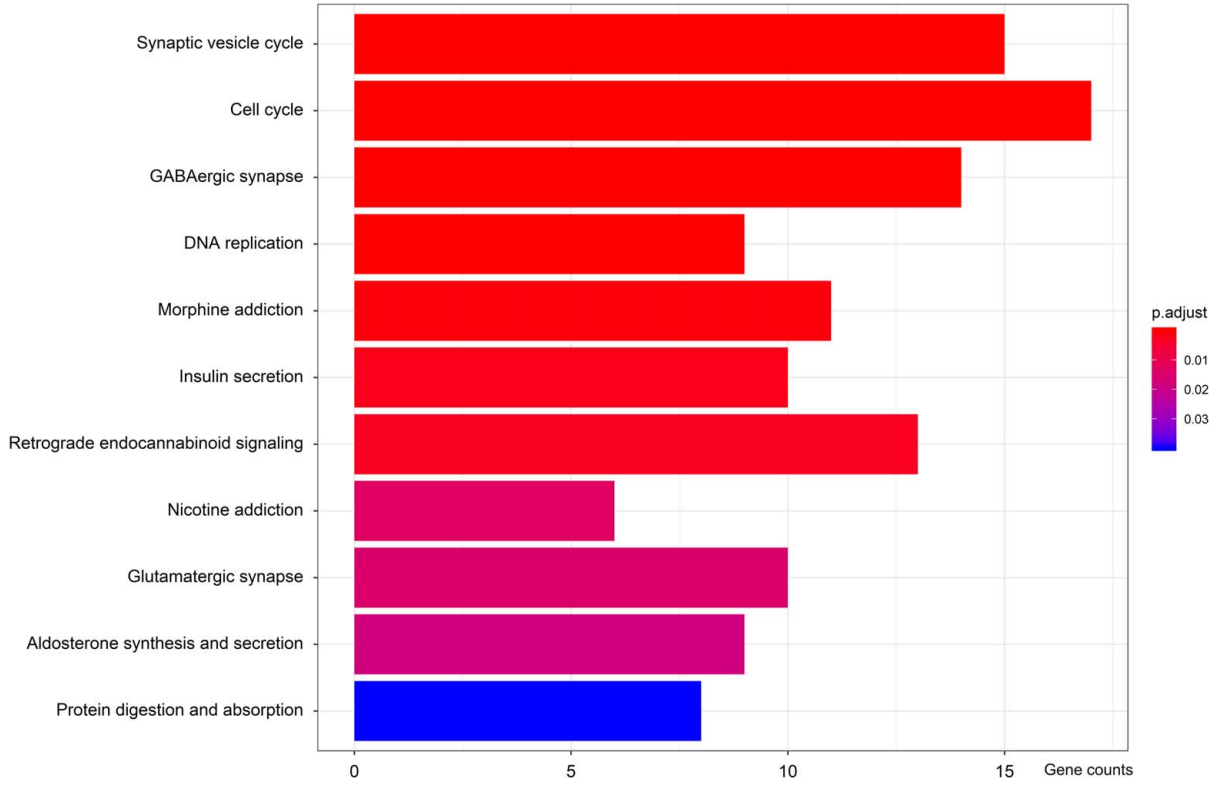

FIGURE 3. KEGG enrichment analysis of DEGs in PNET（the smaller *P* value, the redder color).

TABLE 2. Enriched Signaling Pathways Identified Through KEGG Pathway Analysis

ID	Term	Gene Count	*P*	Gene Names
hsa04721	Synaptic vesicle cycle	15	5.97E-10	SLC17A7, STX1A, CPLX1, ATP6V1G2, SNAP25, DNM1, SLC6A1, STXBP1, RAB3A, SYT1, DNM3, ATP6V0E2, VAMP2, CPLX2, ATP6V1B2
hsa04110	Cell cycle	17	9.61E-09	YWHAG, MCM7, MCM5, E2F5, HDAC1, MCM3, PCNA, CDK4, WEE1, CCNB1, CCND1, MCM2, BUB1B, MAD2L1, PTTG1, CDC20, TTK
hsa04727	GABAergic synapse	14	3.45E-08	GABBR2, ADCY1, GNAO1, GNG3, KCNJ6, SLC12A5, GABRB1, SLC6A1, GABRD, GABRG2, GABBR1, GABARAPL1, GABRB3, GABRA1
hsa03030	DNA replication	9	1.67E-07	RPA3, RNASEH2A, MCM7, MCM5, MCM3, POLE2, PCNA, RFC4, MCM2
hsa05032	Morphine addiction	11	1.49E-05	GABBR2, ADCY1, GNAO1, GNG3, KCNJ6, GABRB1, GABRD, GABRG2, GABBR1, GABRB3, GABRA1
hsa04911	Insulin secretion	10	5.14E-05	CAMK2B, STX1A, ADCY1, SNAP25, ATP1B1, ABCC8, RAB3A, ATP1B2, VAMP2, GNA11
hsa04723	Retrograde endocannabinoid signaling	13	7.92E-05	SLC17A7, ADCY1, GNAO1, GNG3, KCNJ6, GABRB1, GABRD, MGLL, GRM1, GABRG2, GABRB3, GABRA1, ITPR1
hsa05033	Nicotine addiction	6	0.00042726	SLC17A7, GABRB1, GABRD, GABRG2, GABRB3, GABRA1
hsa04724	Glutamatergic synapse	10	0.00053959	SLC17A7, ADCY1, GNAO1, GRM3, GNG3, GRM4, SHANK3, GRM1, SHANK2, ITPR1
hsa04925	Aldosterone synthesis and secretion	9	0.0007322	CAMK2B, ADCY1, ATP2B2, ATP1B1, ATP1B2, AGT, ITPR1, NR4A2, GNA11
hsa04974	Protein digestion and absorption	8	0.00177621	SLC8A2, ATP1B1, PRSS3, ATP1B2, COL4A1, COL3A1, COL11A1, COL5A2

KEGG indicates Kyoto encyclopedia of genes and genomes.

the connectivity degrees were implemented through Cytoscape. Screening of the Small Molecular Compounds Related to PNET

CMap (https://www.broad.mit.edu/cmap), a web-based tool, can produce biological connections among genes, drugs, and diseases based on gene expression profiles.[12] To screen drug candidates, the upregulated and downregulated DEGs were uploaded to CMap. The cutoff criteria were set as follows: $P < 0.005$ and Enrichment <-0.3. For the small molecule compounds, we obtained 3D structures from the Pubchem database (https://pubchem.ncbi.nlm.nih.gov/).

RESULTS

Identification of DEGs in PNET

Two gene expression datasets GSE14295 and GSE74195 were downloaded from GEO. A total of 2019 DEGs were identified from the GSE14295 dataset, including 544 upregulated genes and 1475 downregulated genes. Among the 2013 DEGs identified from GSE74195 dataset, 1154 DEGs were upregulated and 859 DEGs were downregulated. Compared with normal tissues, there were altogether 468 DEGs (including 161 upregulated DEGs and 307 downregulated DEGs) consistently expressed in PNET tissues (Fig. 1 and Table A.1, Supplemental Digital Content 1, http://links.lww.com/JPHO/A591). With reference to GSE74195, a heatmap of DEG distribution was created (Fig. A.1, Supplemental Digital Content 1, http://links.lww.com/JPHO/A591).

Gene Ontology and KEGG Pathway Enrichment Analysis

To further understand the identified DEGs, we performed GO function analysis using DAVID with $P < 0.05$ as the cutoff standard to identify the functions (Table 1). GO function annotation included the following categories: cellular components (CC), molecular function (MF), and biological process (BP). As shown in Figure 2, GO function analysis results showed that for CC, the DEGs were mainly enriched in cytoskeleton, spindle, synaptic vesicle, clathrin-coated vesicle, coated vesicle, neuron projection, axon, synapse, synapse part, and synaptosome. For MF, the DEGs were mainly enriched in GABA A receptor activity, syntaxin binding, tubulin binding, microtubule binding, snare binding, syntaxin-1 binding, motor activity, microtubule motor activity, and structural constituent of cytoskeleton. In addition, BP analysis also displayed that the DEGs were significantly enriched in the nuclear division, organelle fission, cell cycle phase, mitotic cell cycle, mitosis, M phase of the mitotic cell cycle, M phase, cell division, synaptic transmission, and microtubule-based process.

Subsequently, the cellular signaling pathways of 468 DEGs were examined by KEGG. According to KEGG pathway enrichment analysis, the DEGs were predominantly associated with DNA replication, cell cycle, retrograde endocannabinoid signaling, synaptic vesicle cycle, GABAergic synapse, glutamatergic synapse, cell cycle, morphine addiction, nicotine addiction, insulin secretion, aldosterone synthesis and secretion (Fig. 3 and Table 2).

PPI Network Construction and Hub Gene Selection

STRING was used to explore the interrelationships among the DEGs. According to the specific interaction information provided by STRING, 467 of the 468 DEGs were revealed related to each other, and the PPI network,

including 467 nodes and 1156 edges, was visualized using Cytoscape. Based on the degree of connectivity, hub genes were identified (Fig. 4A), and the top-ranked hub genes such as CCNB1, CDC20, KIF11, KIF2C, and MAD2L1 are shown (Fig. 4B).

Potential Small Molecule Drugs for PNET

We converted 453 of the 468 DEGs into IDs based on the Affymetrix platform and then analyzed them with CMap to obtain potential small molecule drugs for PNET. Ten small molecule compounds identified by using set conditions are listed in the Table 3. Among them, trichostatin A has been reported to present an association with PNET, and the other 6 drugs-luteolin, repaglinide, clomipramine, lorglumide, vorinostat, and resveratrol-have been demonstrated to exert tumor-suppressive effects in many cancers. The 3D structures of the small molecule compounds were downloaded from PubChem, except for logludamide, which is not shown due to its too flexible structure (Fig. 5).

DISCUSSION

Early diagnosis, as well as a timely effective therapy, is critical to the patients' survival and prognosis. Although some studies on PNET have been available, conducting a large-scale clinical trial is quite difficult because of the rarity of this disease. Therefore, it is particularly effective to use the existing data to explore key genes related to PNET and small molecular compounds that can be used for the treatment of PNET. In the present study, to screen them, we integrated 2 gene profile datasets downloaded from the GEO database (GSE14295 and GSE74195) and performed a series of bioinformatics analyses. Four hundred sixty-eight DEGs-161 upregulated and 307 downregulated were identified between the tumor and normal samples. To further elucidate the potential functions of these DEGs, GO enrichment, KEGG pathway, and PPI network analysis were also applied. Subsequently, based on the identified DEGs, CMap was used to explore small molecule compounds, and 7 compounds demonstrated to be associated with antitumor effects were eventually screened out as potential therapeutic drugs of PNET.

Results from the GO analysis revealed that the DEGs were significantly enriched in the 3 ontology processes, including BP, CC, and MF. By reviewing the literature, most of these enriched terms have been reported to be closely associated with the progression of cancer, and interference with these processes might execute the antitumor role. For example, in terms of BP, Kollareddy et al[13] reported that YK-4-279 can induce neuroblastoma cell death by promoting mitotic arrest in prometaphase. The function of boron neutron capture therapy targeting tumor is based on regulating the nuclear capture and fission reactions.[14] Anchored on tubulin to inhibit cycles of organelle fission, syntaphilin can block the bioenergetics of prostate adenocarcinoma cell motility and invasion,[15] and knockdown of PRC1- a microtubule-associated protein in oral squamous cell carcinoma leaded to G2/M phase arrest and subsequently blocked cell proliferation and tumor growth.[16] Around CC, Fife et al[17] reported that different components of the cytoskeleton, such as actin and MT, communicated to involve in tumor cell migration and metastasis. Alterations of a synaptic vesicle can affect the chemotherapeutic outcomes of MB.[18] Disturbing the clathrin-coated vesicles internalization of CXCR3-A can increase the migration of glioma cells, and inducing cell spindle defects can strengthen the role of MiR-584-5p in the radiosensitivity of medulloblastoma.[19,20] In addition, focusing on MF, high-level expression of syntaxin binding protein 4 (Stxbp4) can regulate the APC/C-mediated accumulation of ΔNp63 and lead to cell death in squamous cell carcinoma.[21] Endogenous GABA A receptor activity has a marked impact on glioma development by inhibiting tumor cell proliferation and decreasing tumor growth.[22] Targeting

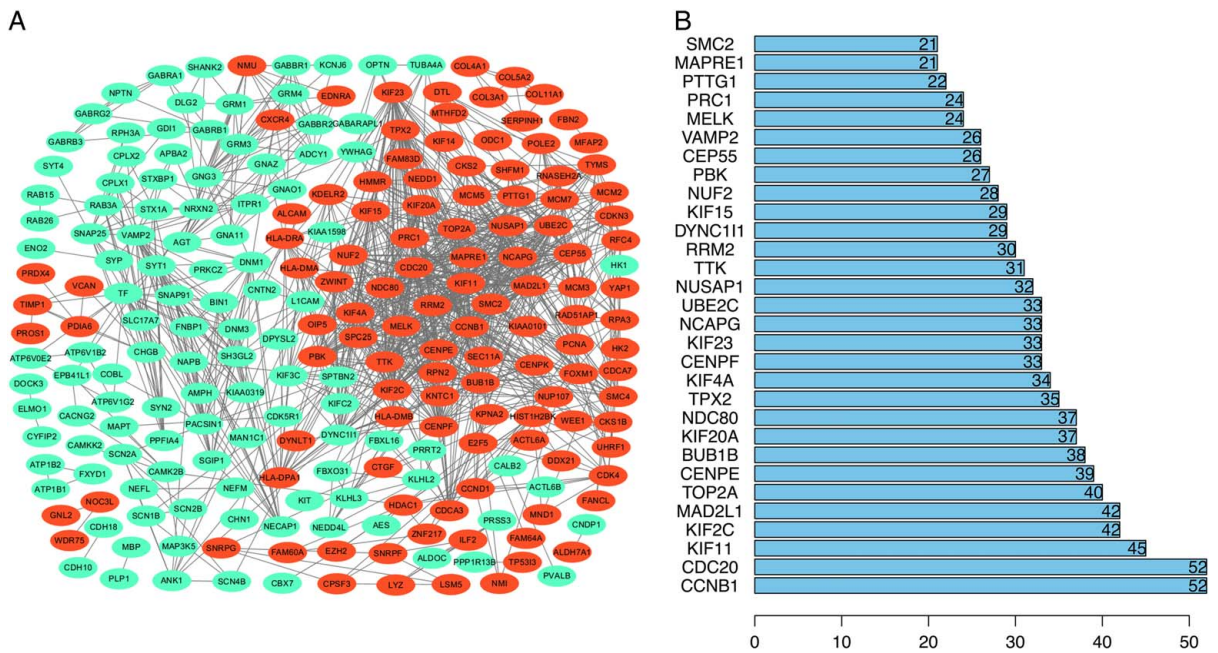

FIGURE 4. PPI network constructed by STRING. (A) 467 DEGs were related to others (red: upregulated genes; green: downregulated genes); (B) Hub genes identified based on the degree of connectivity.

TABLE 3. Ten Small Molecule Compounds Satisfying the Condition Based on the DEGs by CMAP Analysis

Term	Enrichment	*P*
Trazodone	−0.931	0.0005
Luteolin	−0.85	0.00093
Medrysone	−0.807	0.00014
Repaglinide	−0.798	0.00328
Clomipramine	−0.786	0.00418
Lorglumide	−0.73	0.00302
Vorinostat	−0.606	0.00004
Resveratrol	−0.572	0.00256
Prochlorperazine	−0.418	0.00495
Trichostatin A	−0.387	0

CMAP indicates Connectivity map; DEGs, differentially expressed genes.

tubulin binding molecular can exhibit a toxic effect on cancer cells.[23] Microtubule binding molecular also have been reported to participate in the progress of various cancers, including breast cancer, ovarian cancer, and non-small cell lung cancer.[24] These studies indirectly confirmed our analysis results, improving the credibility of our results and the significance of this research.

Moreover, according to KEGG analysis, the DEGs were enriched in 11 KEGG pathways. These enrichment pathways have been reported in previous cancer-related research. For example, when considering methods to treat tumors, it is important to take advantage of the difference in DNA replication stress between tumors and normal cells.[25] The cyclin-dependent kinase (CDK)-RB-E2F axis driving cell cycle progression can lead to uncontrolled proliferation in virtually all cancers.[26] Regulating endocannabinoid systems signaling pathway can block cell divisions and inhibit glioma tumor proliferation,[27] and disturbing synaptic vesicle cycle pathway can induce cell differentiation and then influence the growth of neuroblastoma.[28] In 1 word, studying the enriched functions and pathways enhances our knowledge of PNET and should be useful for the development of novel therapeutic strategies.

Furthermore, PPI network was established to show the interactions among the DEGs, and 5 genes (including CCNB1, CDC20, KIF11, KIF2C, and MAD2L1) were selected as hub genes which might play critical roles in the development of PNET. Other researchers have found that these hub genes were involved in the development and progression of cancer. Cyclin B1 (CCNB1) is an important regulator in the cell cycle and displays a key role in regulating and synthesizing complexes with CDK1 to facilitate the transition from G2 phase to mitosis in the cell cycle.[29] CDC20, a cell-division cycle protein, is an anaphase-promoting complex activator. Knockdown of CDC20 inhibits Wnt signaling through conducting and blocking colony formation of cells in colorectal cancer.[30] KIF2C plays a carcinogenic effect in non-small cell lung cancer and is affected negatively by miR-325-3p.[31] KIF11 is an evolutionarily conserved microtubule motor protein and has been reported to affect MAPK/ERK and PI3K/AKT signaling pathways in breast cancer by interacting with specific kinase or phosphatase.[32] MAD2L1, a mitotic arrest deficiency protein, is a metaphase-anaphase checkpoint member which can affect mitotic-linked cell death through functional p53-p21 signaling in neuroblastoma cells.[33] Although the roles of these genes in cancer have been studied, there is no research available on the correlation with PNET. Therefore, these hub genes might serve as suitable biomarkers for PNET.

In addition, by using CMap online tools, we screened out 10 small molecule drugs, 7 of which have been reported to exhibit antitumor effects. Luteolin can decrease the proliferation, migration, and invasion of glioblastoma cells by blocking the RNA-binding function of Musashi1.[34] Repaglinide, as a putative FOXO3 inhibitor, silences the transcriptional activity of FOXO3, which contributes to poor prognosis of advanced neuroblastoma by promoting chemoprotection and tumor angiogenesis.[35] Clomipramine can enhance the cytotoxicity and apoptosis of human neuroblastoma cancer cell line SH-SY5Y induced by vinorelbine.[36] Desmethylclomipramine, the active metabolite of Clomipramine, can block the growth of lung CSCs, reduce the stemness potential and enhance the cytotoxic action of conventional chemotherapy drugs.[36] Lorglumide has the effect of inhibiting the proliferation of human colon cancer cells, and melatonin can enhance its effect.[37] Vorinostat has radiosensitizing properties and can strengthen the antitumor effect combined with other drugs in the treatment of tumors such as glioblastoma, and hepatocellular carcinoma cells.[38,39] Resveratrol and As_2O_3 combination therapy can more effectively inhibit the cell viability of human neuroblastoma, which is related to the increase of reactive oxygen species levels.[40] Treatment with 5-Aza-2'-deoxycytidine (5AZA) combined with trichostatin A successfully reestablished RASSF1A expression

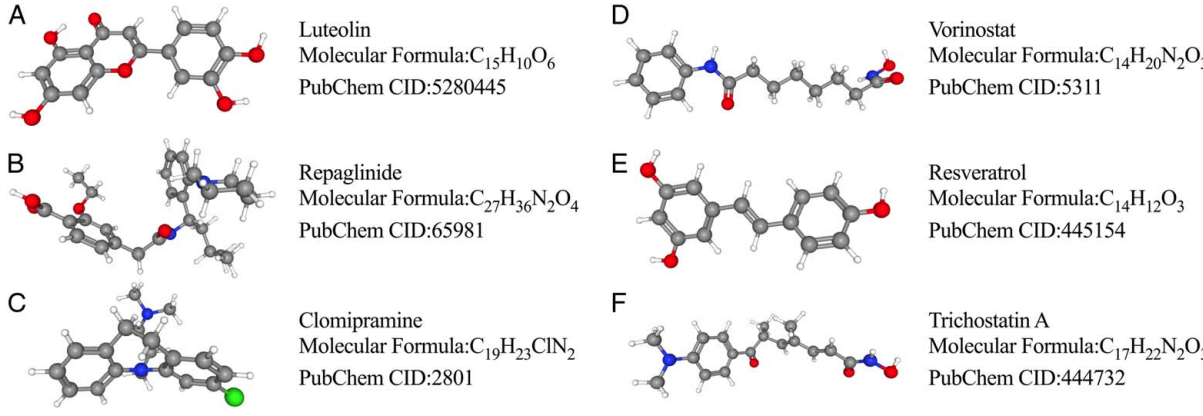

FIGURE 5. Three-dimensional (3D) structures of small molecular compounds identified as the potential drugs for treatment of PNET. (A) Luteolin; (B) Repaglinide; (C) Clomipramine; (D) Vorinostat; (E) Resveratrol; (F) Trichostatin A.

in medulloblastoma cell lines, while abnormal methylation of the RASSF1A promoter was detected in most sPNET.[41]

The evidences above suggest that the small molecule compounds we obtained have the effect of inhibiting tumor growth, and PNET is also a malignant tumor, so it is possible that these drugs have similar effects on PNET and these research results need to be further confirmed. Our research only provides an index, and we hope that more researchers will pay attention to the new effects of some traditional medicines. New usage of old drugs has many advantages, such as a better understanding of the physical and chemical properties and pharmacokinetics of the drugs. These advantages prompt them to be available for clinical use to reduce the patient's suffering as soon as possible. There are many examples regarding the new usage of traditional medicines such as metformin, aspirin, and others. Metformin, as a traditional hypoglycemic drug, has been found to have an inhibitory effect on tumors in recent years, and there are even reports showing that it has anti-inflammatory effects.[42,43] Again, as a traditional antipyretic and analgesic drug, aspirin has an obvious anti-platelet aggregation effect function when used in small doses and then protect cardiovascular.[44]

Thus, we firmly believe that although there is no direct evidence that the small molecule compounds we obtained have an inhibitory effect on PNET, we have sufficient reasons to support them as potential effective drugs and to draw more attention of many researchers in the research for their new effects. In addition, our study still has some limitations. First, the classification of central nervous system tumors has undergone many changes due to the discovery of genotypes. Precise analysis of PNET with specific genotypes or pathological types is needed to find more effective therapeutic targets and drugs in the future; second, the number of samples obtained from the GEO database is limited, and data analysis based on small samples may cause biased results; again, although microarray-based bioinformatics analysis is a powerful tool to effectively understand molecular mechanisms and identify potential biomarkers, further research is still needed. Finally, whether the screened potential small molecule compounds are effective against the disease still needs experimental verification and prospective clinical research.

CONCLUSION

In this study, the integration of multiple data sets was exploited to enlarge the sample size and further improve the reliability of the results. By multiple microarray analysis of the downloaded gene expression profile data, 468 DEGs, enriched biological functions and pathways, and 5 hub genes were identified, which all may exert potential functions in PNET, and these hub genes, including CCNB1, CDC20, KIF11, KIF2C, and MAD2L1, may serve as key markers of PNET. In addition, the present study screened small molecule compounds to identify 7 new drug candidates, including trichostatin A, luteolin, repaglinide, clomipramine, lorglumide, vorinostat, and resveratrol, but further studies are still required to investigate their therapeutic functions on PNET and ascertain the molecular mechanisms of the actions.

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