Original Article

Screening for biomarkers of tuberous sclerosis complex–associated epilepsy: a bioinformatics analysis

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Background: The optimal biomarkers for early diagnosis, treatment, and prognosis of tuberous sclerosis complex (TSC)-associated epilepsy are not yet clear. This study identifies the crucial genes involved in the pathophysiology of TSC-associated epilepsy via a bioinformatics analysis. These genes may serve as novel therapeutic targets.

Methods: Gene chip data sets (GSE62019 and GSE16969) comprising the data of patients with TSC-associated epilepsy and healthy control participants were obtained from the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) in the GEO database were identified using the GEO2R gene expression analysis tool. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Gene Ontology function, and protein-protein interaction (PPI) network analyses were then conducted. The results were analyzed using R language, and are presented in volcano plots, Venn diagrams, heatmaps, and enrichment pathway bubble charts. A gene set enrichment analysis (GSEA), was conducted to examine the KEGG pathways and crucial genes linked to TSC-associated epilepsy. The potential genes were compared with the genes listed in the Online Mendelian Inheritance in Man (OMIM) database and analyzed against the literature to determine their clinical significance. Finally, the expression of the key genes in the TSC-associated epilepsy mice cerebral cortex was examined through immunohistochemical staining.

Results: The intersection of the GSE62019 and GSE16969 data sets revealed 151 commonly upregulated DEGs. The KEGG enrichment analysis indicated that these DEGs affected the occurrence and development of TSC-associated epilepsy by modulating complement and coagulation cascades, glycosaminoglycans in cancer, and extracellular matrix-receptor interactions. Four high-scoring clusters emerged, and podoplanin (*PDPN*) was identified as a key gene through the construction of a PPI network of the common DEGs using the Cytoscape software. A GSEA of the DEGs revealed that the common DEG *PDPN* was enriched in both data sets in pathways related to platelet activation, aggregation, and the glycoprotein VI (GPVI)-mediated activation cascade. Immunohistochemical staining revealed a significant elevation in *PDPN* expression in the cerebral cortex of mice with TSC-associated epilepsy. Conversely, the control group mice did not display any significantly positive neurons.

Conclusions: The discovery of these crucial genes and signaling pathways extends understanding of the molecular processes underlying the development of TSC-associated epilepsy. Additionally, our findings may provide a theoretical basis for research into targeted clinical treatments.

Keywords: Tuberous sclerosis complex (TSC); epilepsy; podoplanin (PDPN); bioinformatics; prognosis

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Introduction

Tuberous sclerosis complex (TSC) is a genetic disorder manifesting as an autosomal dominant neurocutaneous syndrome that affects many tissues or organs concurrently or sequentially, including the skin, lungs, kidneys, heart, and central nervous system (CNS). Approximately 22.6% of individuals with TSC have a family history of the condition (1). Mutations in the *TSC1* gene, which encodes hamartin on chromosome 9, and/or the *TSC2* gene, which encodes tuberin on chromosome 16, are presently thought to be associated with TSC (2). However, mutations of these genes are not found in 14.4% of patients with TSC (1).

The mammalian target of rapamycin (mTOR) is an essential mediator of cell proliferation and a serine/threonine kinase that is exceptionally conserved among eukaryotes. It is implicated in protein synthesis, gene transcription and translation, and ribosome formation (3). During the embryonic phase, the hyperactivation of the mTOR signaling pathway, driven by mutations in the *TSC1* and/or *TSC2* genes, leads to the formation of hamartomas. This hyperactivation results in aberrant proliferation, differentiation, and unregulated apoptosis of ectodermal, mesodermal, and endodermal cells. The CNS is the most frequently affected organ, with histopathological manifestations of cortical or subcortical nodules, subependymal nodules, subependymal giant cell astrocytomas, and white matter migration lines (4). These manifestations typically result in clinical symptoms such as epileptic seizures and neurological deficits.

However, there are significant differences in clinical manifestations among patients with different genetic phenotypes. Patients with *TSC2* gene mutations have a younger onset age of epilepsy, and higher rates of focal and refractory epilepsy (5). Even among patients with the same genetic phenotype, clinical manifestations vary greatly due to differences in affected organs. Epileptic seizures typically manifest as infantile spasms and focal seizures, with the latter being the most prevalent, affecting 83.6% of TSC patients of all ages, albeit such seizures are most frequently observed in infancy (6,7). TSC-associated epilepsy is often refractory and requires a combination of treatment approaches. Thus, for the early detection, management, and prognosis of TSC-associated epilepsy, it is critical to leverage bioinformatics methodologies and gene chip technology to systematically identify and analyze novel biomarkers (8).

This study utilizes the Gene Expression Omnibus (GEO) database and employs bioinformatics analysis methods to further explore differentially expressed genes (DEGs), disease-related signaling pathways, and protein-protein interaction (PPI) networks in TSC-related epilepsy. The aim is to identify key genes that may play significant roles in the progression of TSC-related epilepsy at the molecular level, thereby providing a theoretical basis for understanding the molecular mechanisms underlying the disease. We present this article in accordance with the STREGA and ARRIVE reporting checklists (available at https://tp.amegroups.com/article/view/10.21037/tp-24-211/rc).

Highlight box

Key findings

- This study identified key genes and signaling pathways involved in tuberous sclerosis complex (TSC)-associated epilepsy utilizing bioinformatics approaches.

What is known and what is new?

- Previously, TSC was associated with mutations in *TSC1* and *TSC2* genes, leading to mammalian target of rapamycin (mTOR) pathway hyperactivation. However, a significant proportion of TSC patients remain without identifiable genetic variants.
- This study adds to our understanding by revealing novel differentially expressed genes and their involvement in TSC-associated epilepsy. It highlights the role of *PDPN* and associated signaling pathways in disease pathogenesis.

What is the implication, and what should change now?

- Future research should focus on validating the role of identified genes and pathways in disease progression and exploring their potential as therapeutic targets.

Methods

Data sets

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Gene chip data

sets containing the data of patients with TSC-associated epilepsy and normal control patients (GSE62019 and GSE16969) were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). To be selected for inclusion in the study, the data sets had to meet the following inclusion criteria: (I) comprise whole-genome messenger RNA expression chip data; (II) include the data of healthy control participants and patients with TSC-associated epilepsy; (III) have undergone standardization processing; and (IV) include more than three samples per data set.

Data processing and DEG screening

To examine the distribution between groups, a principal component analysis (PCA) was performed on the samples from the two data sets, each classified by chip, using the R programming language. All the DEGs in each data set were evaluated using the GEO2R gene expression analysis tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/). The following selection criteria were used to identify the DEGs: log fold change (FC) ≥1 and $P<0.05$. Venn diagrams were employed to examine the direct intersections between the DEGs in both chips, including those that showed inconsistent upregulation and downregulation, for the subsequent bioinformatics analysis of the related pathways. To exclude the influence of false positive co-expressed genes and to screen for predictive targets for clinical diagnosis and prognosis, genes that are upregulated might offer greater clinical applicability and research significance when compared to those in healthy individuals. Consequently, our analysis focused solely on genes that were consistently upregulated among the DEGs. Heatmaps and volcano plots for the DEGs obtained from the two data sets were generated using R language. Venn diagrams were used to intersect the upregulated DEGs identified from the two data sets and to identify the consistently upregulated DEGs linked to TSC-associated epilepsy.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses and gene set enrichment analysis (GSEA)

KEGG and GO analyses were conducted to explore the biological functions of the DEGs in TSC-associated epilepsy. A GO analysis is a powerful bioinformatics tool for identifying the biological processes (BPs), cellular components (CCs), and molecular functions (MFs) associated with DEGs. A GSEA was performed to analyze the similar potential mechanistic pathways.

PPI network analysis of common DEGs

An evaluation of the PPI network among the common DEGs was carried out using the online Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org/). Subsequently, the obtained results were analyzed and visualized using Cytoscape software (https://cytoscape.org/). Significant protein expression molecules were detected via the Molecular Complex Detection (MCODE) plugin in Cytoscape, an algorithm for clustering protein complexes.

Immunohistochemistry

Data Sharing Statement

Twenty 6–8 weeks old specific-pathogen-free (SPF)-grade male mice were purchased from the Experimental Animal Center of Nantong University (Nantong, China). Based on previous literature (9), we developed a mice model of TSC-associated epilepsy. These TSC mouse models exhibit phenotypic characteristics such as spontaneous seizures, behavioral abnormalities including anxiety and hyperactivity, cognitive dysfunction, and brain nodular sclerosis lesions. The animals were anesthetized with 0.3% pentobarbital sodium via intraperitoneal injection, and then systemically perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) sequentially. Brain tissues were extracted and fixed externally in 4% PFA for 48 hours, and then dehydrated with 30% sucrose. The brain tissues were then sectioned into 30-μm thick frozen slices. Immunohistochemical staining was initiated with blocking in 10% goat serum, incubation with primary antibodies (anti-PDPN, 1:200, ab217886, Abcam, Cambridge, UK), followed by incubation with secondary antibodies. The slides were then mounted for microscopy. Animal experiments were performed under a project license (No. S20210601-204) approved by committee of Nantong University, in compliance with institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration.

Statistical analysis

GraphPad Prism 7.0 software (https://www.graphpad.com/) was used to conduct the statistical analysis. The data are presented as the mean ± standard deviation. A *t*-test was

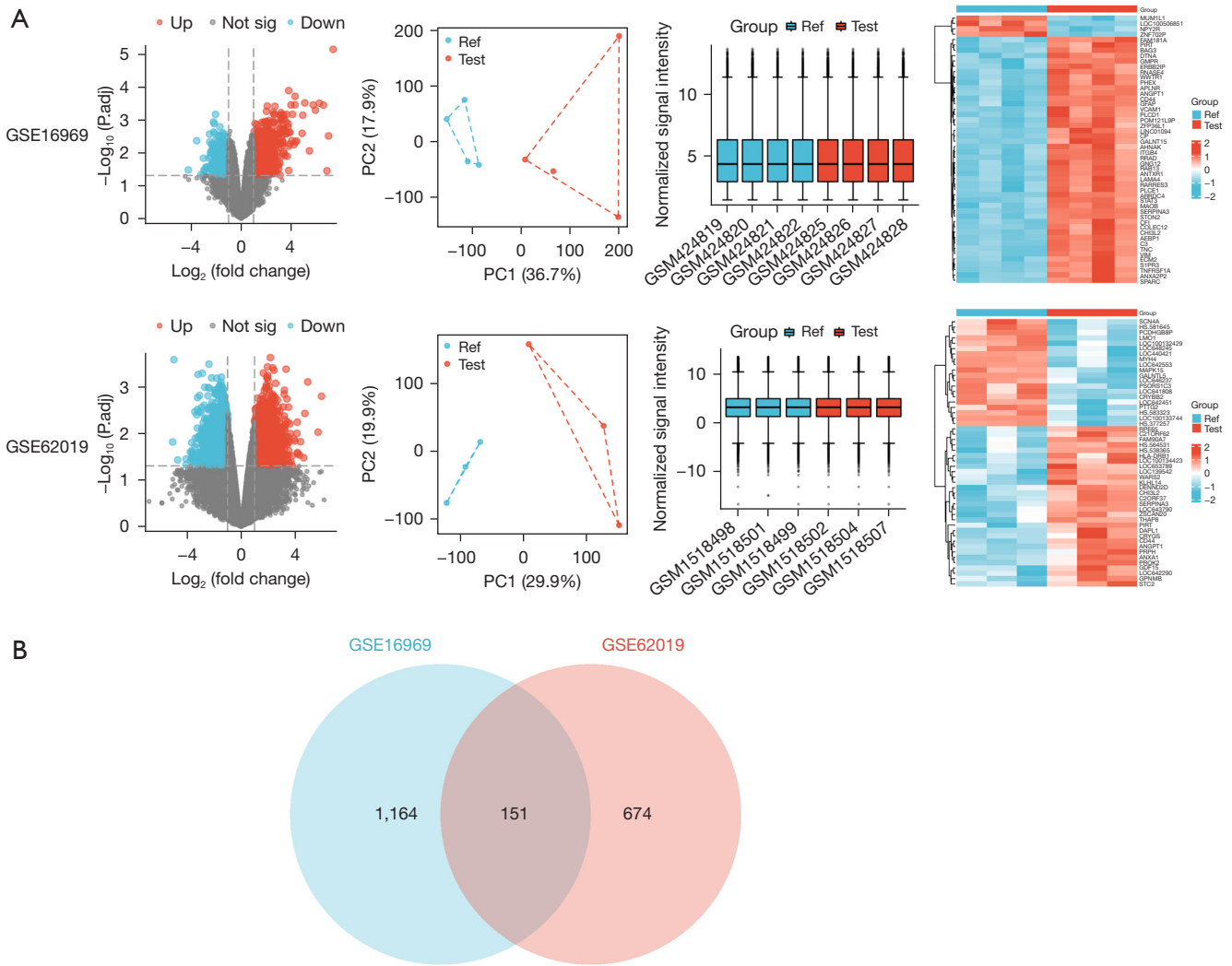

Figure 1 Selection of DEGs associated with TSC-associated epilepsy. (A) Volcano plot, PCA plot, sample normalization box plot, and heatmap of DEGs in the GSE16969 (top) and GSE62019 data sets (bottom). (B) Venn diagram of the upregulated DEGs in both the GSE62019 and GSE16969 data sets. P.adj, adjusted P value; sig, significant; PC, principal component; DEGs, differentially expressed genes; TSC, tuberous sclerosis complex; PCA, principal component analysis.

employed to determine the P value for the DEG analysis, with the false discovery rate adjustment applied to the P values. A P value ≤0.05 was considered statistically significant.

Results

Identification of the DEGs associated with TSC-associated epilepsy

Gene chip data sets comprising the data of TSC-associated epilepsy patients and normal control patients (GSE62019 and GSE16969) were selected from the GEO database. Based on log FC ≥1 and P<0.05 as the screening criteria, volcano plots, PCA plots, sample normalization box plots, and heatmaps for the DEGs were generated (*Figure 1A*). By intersecting the upregulated DEGs from both data sets, we constructed a Venn diagram and identified 151 upregulated DEGs (*Figure 1B*). These specific genes are listed in Table S1.

GO and KEGG enrichment analysis

Enrichment analysis of common DEGs screened using the DAVID (Database for Annotation, Visualization

and Integrated Discovery, https://david.ncifcrf.gov/) online database with a background of *Homo sapiens*, to obtain GO enrichment information. The upregulated DEGs were mainly enriched in BPs such as lymphocyte-mediated immunity, cytokine-mediated signaling pathways, mesenchymal cell differentiation, the positive regulation of ion transmembrane transport, and the regulation of interleukin-8 production. In terms of the CCs, the upregulated DEGs were enriched in the lumen of secretory granules and cytoplasmic vesicles, phagosomes, the peptidase inhibitor complex, and primary lysosomes. For the MFs, the upregulated DEGs were associated with serine-type endopeptidase activity, cell adhesion molecule activity, cadherin binding involved in cell-cell adhesion, Toll-like receptor binding, and the extracellular matrix (ECM) structural constituent. The KEGG pathway enrichment analysis revealed that the upregulated DEGs were primarily related to coagulation and complement pathways, glycosaminoglycans in cancer, and ECM-receptor interactions (*Figure 2*).

Development and module analysis of PPI network of the common DEGs and the selection of the core genes

A PPI network of the common DEGs was generated via STRING. The protein network was then examined by a visual analysis in Cytoscape. The degree values for each node in the network were obtained using the network analyzer tool in Cytoscape to determine the undirected scores. The size of the nodes represented the degreed values, while the color gradient from red to green represented high to low neighborhood connectivity, respectively. The thickness of the edges represented the combined score values. All the protein nodes were arranged using the Attribute Circle layout, with nodes with a degree ≥4 placed in the inner layer (*Figure 3A*). We used the MCODE plugin to cluster and analyze these protein molecules, and employed default parameters such as a node score cut-off value of 0.2, k-score value of 2, and maximum depth of 100, which led to the identification of four high-scoring clusters (*Figure 3B*). Moreover, a search of the Online Mendelian Inheritance in Man (OMIM) (https://www.omim.org/) database for human genes and genetic diseases revealed 454 genes linked to TSC-associated epilepsy in the literature. One candidate gene, podoplanin (*PDPN*), was identified by intersecting the 454 genes with the 70 crucial protein molecules found through the MCODE analysis via a Venn diagram (*Figure 3C*).

GSEA of DEGs

The GSEA uses the KEGG gene set in the C2 dataset as the preset gene set, and conducts core differential gene screening on two chip datasets, with $P<0.05$ as the screening criterion for enrichment pathways related to epilepsy associated with TSC.

Ultimately, we found that the common DEG *PDPN* was enriched in both data sets in the signaling pathways related to platelet activation, aggregation, and the glycoprotein VI (GPVI)-mediated activation cascade (*Figure 4*).

PDPN protein expression in the cortex of mice with TSC-associated epilepsy

The immunostaining results showed bright red intracellular staining in neurons in the cerebral cortex of the model mice, indicating a high level of PDPN in the cortex of mice with TSC-associated epilepsy. Conversely, no substantial positive neurons were found in the brains of the control group mice, which suggest that PDPN may play a crucial role in the development and advancement of TSC-associated epilepsy (*Figure 5*).

Discussion

As an autosomal dominant genetic disorder, TSC is predominantly caused by mutations in the hamartin- and tuberin-encoding tumor suppressor genes *TSC1* (9q34) or *TSC2* (16p13.3). These mutations result in protein deficiencies, aberrant heterodimer formation, and the stimulation of the mTOR complex 1 signaling pathway, which promotes the development of hamartomas in various organs, including the brain, skin, heart, kidneys, liver, and lungs, which in turn adversely affects the functionality of these organs (10,11).

The TSC phenotype displays a wide range of characteristics, of which epileptic seizures are a prevalent neurological symptom and the main reason for patient consultations. Studies have reported that the occurrence rate of seizures in individuals with TSC is 62–93% (12,13), the incidence is similar between both genders, and the onset of epileptic-like seizures typically occurs before the age of 1 year. Seizures may appear as focal events preceding spasms or may occur simultaneously with or progress into spasms (14-16). The main cause of TSC-associated epilepsy is not fully understood.

However, several potential risk factors for TSC-associated epilepsy have been identified, including chromosomal

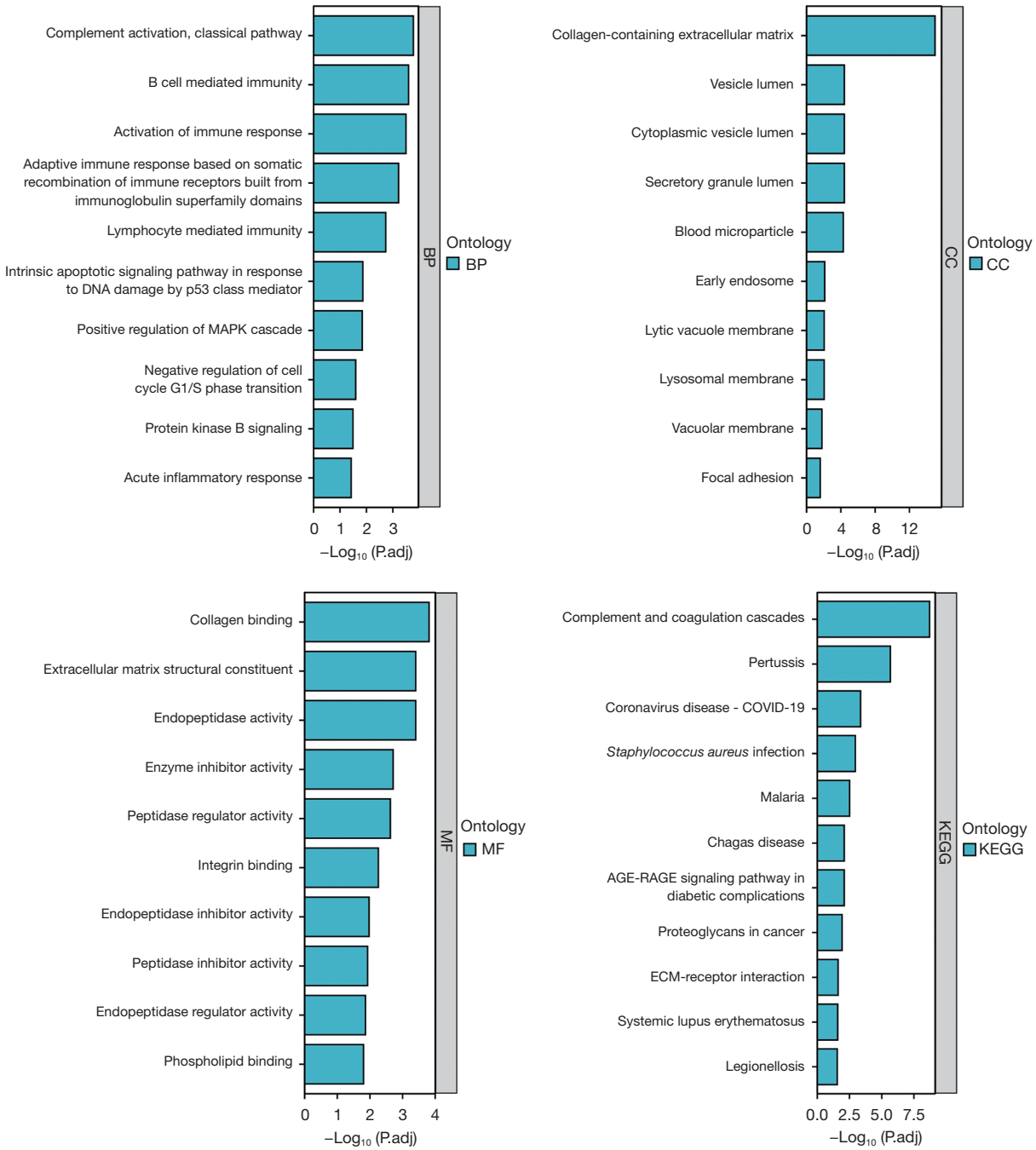

Figure 2 GO and KEGG enrichment analysis results of the upregulated DEGs. P.adj, adjusted P value; MAPK, mitogen-activated protein kinase; BP, biological process; CC, cellular component; MF, molecular function; AGE, advanced glycation end products; RAGE, receptor for AGE; COVID-19, coronavirus disease 2019; ECM, extracellular matrix; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; DEGs, differentially expressed genes.

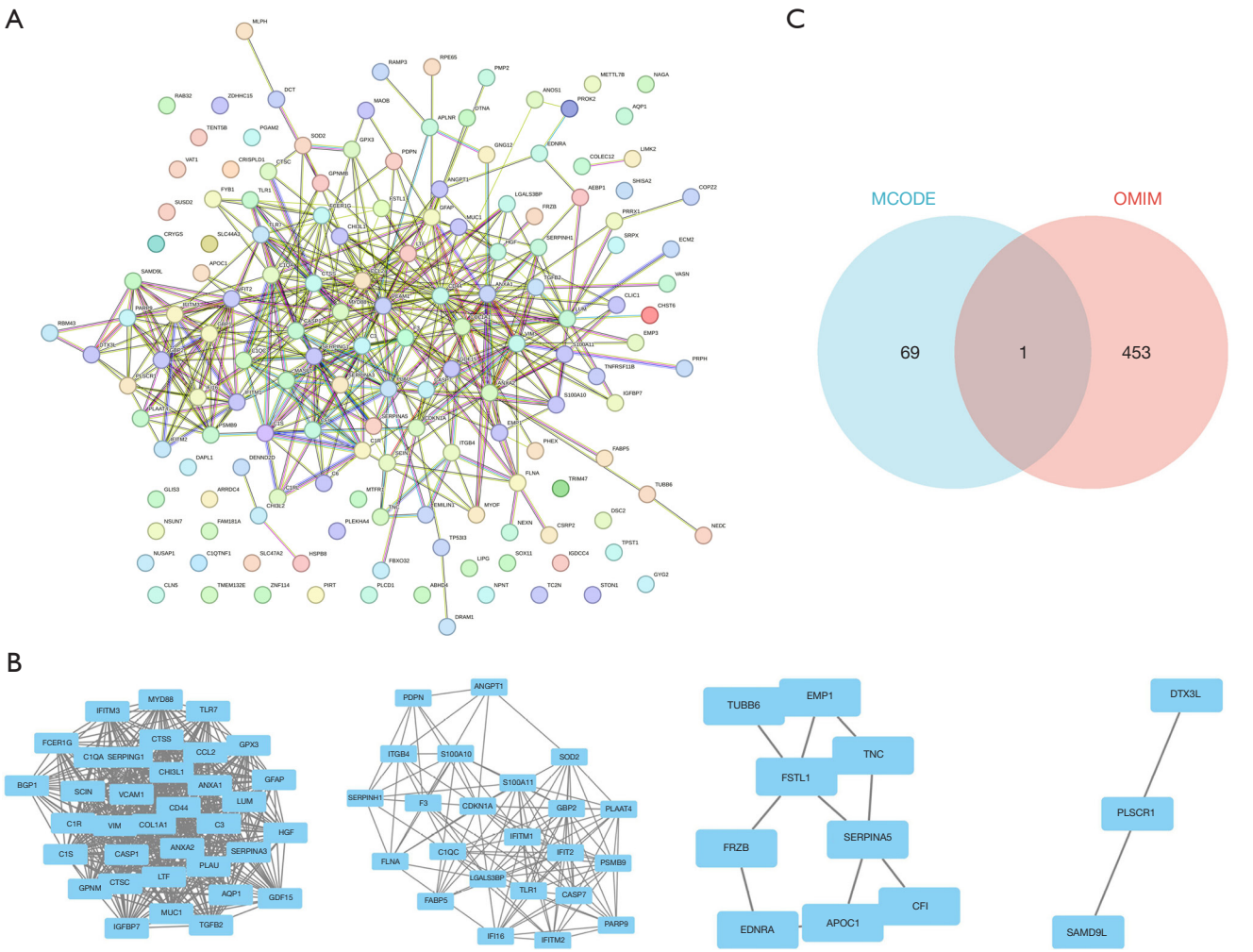

Figure 3 PPI network and candidate gene Venn diagram. (A) PPI network diagram. (B) Four related clusters. (C) Candidate gene Venn diagram. PPI, protein-protein interaction; MCODE, Molecular Complex Detection; OMIM, Online Mendelian Inheritance in Man.

abnormalities, genetic alterations, changes in the cellular microenvironment, and immune regulation (17,18).

Gene chips, which allow for the comprehensive analysis of gene expression profiles, have recently become a widely used tool in the study of various diseases (19). In this study, we aimed to identify DEGs that are consistently upregulated in TSC-associated epilepsy from gene chip datasets in the GEO database using bioinformatics techniques. These DEGs were then subjected to functional enrichment analyses to explore their roles in biological pathways relevant to the disease. The identification of such biomarkers is crucial as it can provide a theoretical foundation for understanding the disease and potentially guide the development of targeted therapies.

In this study, we used bioinformatics techniques to retrieve and analyze data sets related to TSC-associated epilepsy from the GEO database. Our analysis identified 151 upregulated common DEGs. The DAVID online tool was used to perform GO and KEGG enrichment analyses to investigate the biological roles of the DEGs. The KEGG signaling pathway enrichment analysis revealed that the overexpressed DEGs were primarily associated with complement and coagulation pathways, glycosaminoglycans in cancer, and interactions between ECM receptors.

Previous studies have reported similar findings, including a negative correlation between cortical glucose metabolism in TSC-associated epilepsy and delta-slowing and peak frequency (20), that histone deacetylase inhibitors can

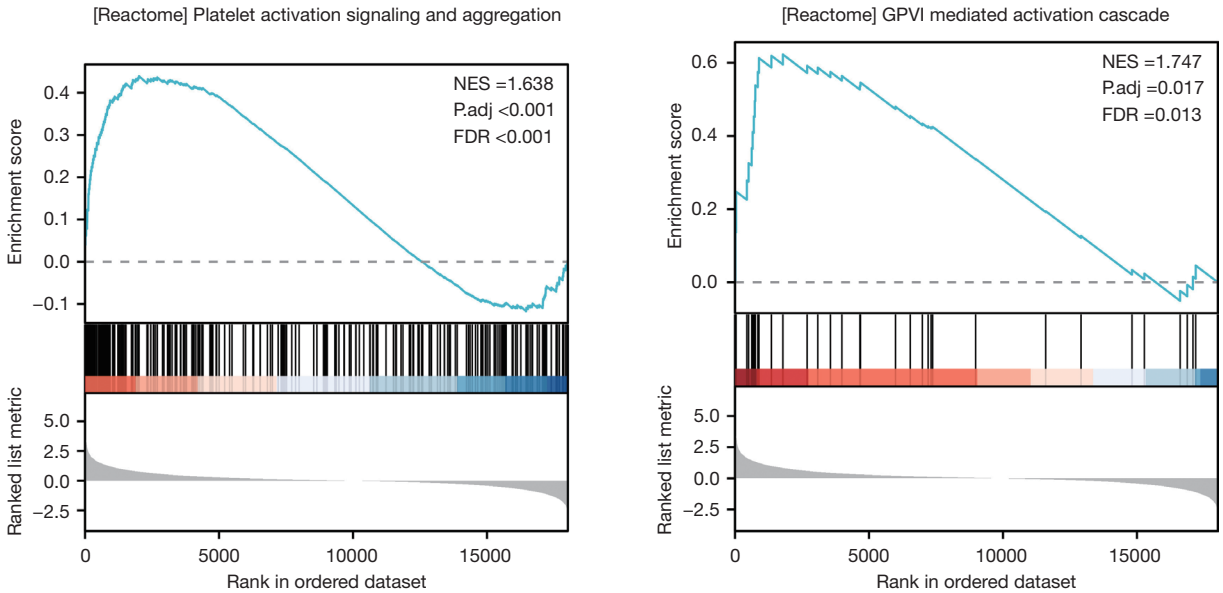

Figure 4 Enrichment results of the KEGG preset gene sets for the GSEA. NES, normalized enrichment score; P.adj, adjusted P value; FDR, false discovery rate; GPVI, glycoprotein VI.

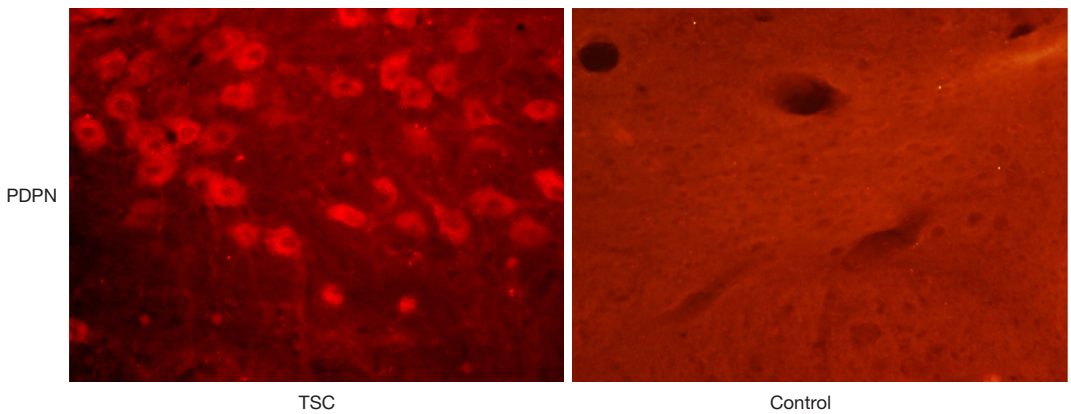

Figure 5 Immunostaining results. High-intensity red-positive immunoreactive substances in the neuronal cell bodies in the cortexes of mice with TSC-associated epilepsy, which indicated the high expression of the PDPN protein (40×). PDPN, podoplanin; TSC, tuberous sclerosis complex.

restore normal synaptic plasticity in the hippocampus and increase the seizure threshold in mice models of TSC (21), and that adenosine analogue AppCH2ppA inhibits seizures by stimulating adenosine signaling in the cortex (22). These findings indirectly support the accuracy and research value of our study.

The *PDPN* gene, which was identified using Cytoscape software, encodes a protein that is expressed in various tissues and cell types, including tumor and immune cells. It is implicated in various processes that contribute to the formation and progression of tumors, including angiogenesis, metastasis, and tumor cell migration. Previous research has shown that *PDPN* expression levels are positively correlated with glioma grading in diffuse astrocytomas (23). High *PDPN* expression has also been found to be associated with lower survival rates in patients with astrocytomas with mutated or wild-type isocitrate dehydrogenase (*IDH*) and in patients with glioblastoma with

wild-type *IDH*. Research has shown that the knockdown of *PDPN* hinders the growth of glioma cells and reduces the levels of phosphorylated Akt (protein kinase B) and phosphorylated mTOR proteins (23). The overexpression of *PDPN* is correlated with mTOR pathway activity and stimulates the mTOR pathway, resulting in enhanced growth, infiltration, and metastasis of tumor cells. Further, the activation of the mTOR pathway also induces tumor cells to express more *PDPN*, forming a positive feedback loop that further promotes tumor development (24,25). Collectively, these findings reflect the research value of *PDPN* in TSC-associated epilepsy.

The GSEA of the DEGs revealed that the common DEG *PDPN* was enriched in both data sets in the signaling pathways related to platelet activation, aggregation, and the GPVI-mediated activation cascade. These pathways have also been implicated in epilepsy. Notably, Kopeikina *et al.* (26) reported that platelets were significantly enhanced in a pentylenetetrazol-induced seizure mouse model. Platelets have also been found to actively secrete serotonin, a neurotransmitter that facilitates enhanced blood-brain barrier (BBB) permeability and has been detected in the CNS during seizures. Further, research has shown that platelets induce oxidative stress in neurons by directly stimulating neuronal electrical activity and inducing the expression of genes associated with early neuronal reaction, neuroinflammation, and oxidative phosphorylation (26). Kopeikina *et al.* (27) found that platelet activation in the CNS occurs when platelets recognize significant brain gangliosides on the surfaces of neurons and astrocytes. This activation results in the release of neurotransmitters and pro-inflammatory neurotrophic factors. Platelet-derived factors promote the formation of new synapses and axonal regeneration toward the injury site by directly stimulating neuronal electrical and synaptic activities. The disruption of the BBB may be closely related to neuronal damage during epilepsy and psychiatric disorders. Additionally, we identified high levels of *PDPN* expression in the cerebral cortex of the model mice via immunohistochemistry; however, no significantly positive neurons were observed in the cerebral cortexes of the control group mice. These results suggest that *PDPN* may have a significant effect on the progression and onset of TSC-associated epilepsy. Our findings may also provide a foundation for future *in vitro* and *in vivo* studies.

One significant limitation of this study is the potential for confounding factors that were not accounted for in the analysis. Since the comparison was made between patients with TSC-associated epilepsy and healthy controls, it remains unclear whether the observed upregulation of *PDPN* and other DEGs are specifically associated with TSC-associated epilepsy or if they might also be influenced by other factors such as general epilepsy, the effects of antiseizure medication, TSC-associated neuropsychiatric disorders, or other common manifestations of TSC. To address this limitation, future research should include additional control groups, such as patients with non-TSC epilepsy and those with other TSC manifestations without epilepsy, and those with sporadic focal epilepsies (28). These comparisons will help to disentangle the specific contributions of these genes to the pathophysiology of TSC-associated epilepsy. Furthermore, functional studies are needed to validate the roles of the identified genes and pathways in TSC-associated epilepsy and to explore their potential as therapeutic targets.

Conclusions

Several regulatory factors influence the progression of TSC-associated epilepsy. The identified key genes and signaling pathways provide insights into the molecular processes involved in the development of TSC-associated epilepsy. Further, these findings may provide a theoretical basis for research into targeted clinical treatments.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the STREGA and ARRIVE reporting checklists. Available at https://tp.amegroups.com/article/view/10.21037/tp-24-211/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-211/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-24-211/coif). P.S. has received consulting fees from Proveca, Italfarmaco, Jazz, UCB, speaker fees from Biomarin, Neuraxpharma, and participated on advisory board for Proveca, outside the submitted work. G.P.W. has received speaker fees from Paladin and Sunovion, and participated on advisory boards for Jazz Pharma and Paladin, outside the submitted work. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Animal experiments were performed under a project license (No. S20210601-204) approved by committee of Nantong University, in compliance with institutional guidelines for the care and use of animals.

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