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Screening and identification of key biomarkers for retinoblastoma

Evidence from bioinformatics analysis

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Abstract

Background: Retinoblastoma (RB) is one of the most common malignant tumors in pediatrics; to clarify the cause of RB, a lot of manpower and material resources have been invested but have not been well explained.

Methods: To identify the candidate genes in the occurrence and development of the disease, we downloaded the microarray datasets GSE97508, GSE92987, and GSE24673 from the gene expression database (GEO). The differentially expressed gene (DEG) was identified and functional enrichment analysis was performed. The protein–protein interaction network was constructed and analyzed by String and Cytoscape.

Results: A total of 74 DEGs were identified, including 40 up-regulated genes and 34 down-regulated genes. The rich functions and pathways of DEG include regulating mitosis, cell cycle, DNA transcription process, promoting protein phosphorylation, regulating energy metabolism in vivo, promoting the binding of some macromolecular complexes, and regulating the cell cycle. Twenty-four HUB genes were identified. Biological process analysis showed that these genes were mainly enriched in regulating energy metabolism in vivo, promoting the binding of some small molecules and regulating the cell cycle. Survival analysis showed that DGPDC1, NDC80, SHCBP, TOP2A, and DLGAP5 may be involved in the occurrence, invasion, or recurrence of RB.

Conclusion: In conclusion, screening DEGs and HUB genes in RB can help us to better understand the mechanism of the occurrence and development of RB at the molecular level, and provide candidate targets for the diagnosis and treatment of RB.

Abbreviations: DEGs = differentially expressed gene, GEO = gene expression database, GO = Gene Ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, PPI = protein–protein interaction network, RB = retinoblastoma.

Keywords: bioinformatics, biomarkers, retinoblastoma

1. Introduction

Retinoblastoma (RB) is the most common ocular malignant tumor in infants and young children, accounting for about 4% of all malignant tumors in children. The incidence of the disease is about 1:18000 to 1:21000.[1] The number of new children in China is about 1100 to 1500 cases per year. In Europe and the United States, the 5-year survival rate of children with this disease has exceeded 95%, whereas the global average 5-year survival rate, including developing regions, is about 50%.[2] Early diagnosis and standardized treatment of RB have always been the focus of ophthalmic tumor research. At present, there is no effective marker to evaluate the prognosis of ophthalmic tumors. More and more evidence show that gene mutation leads to abnormal expression of the protein, which plays an important role in the occurrence and development of RB and tumor suppressor gene mutation. Studies have shown that MALAT1 can regulate the proliferation and development of RB SO-Rb50 cell line. LncRNA plasma cell tumor variant translocation 1 is a highly expressed oncogene in a variety of tumors, which is upregulated in RB tissues and cell lines, resulting in a poor overall survival rate. After silencing or knockout of this gene, cell proliferation, migration, invasion, and cell cycle processes were significantly inhibited, inducing apoptosis, and the expression of Notch2 was also inhibited.[3] ACVR1C/Smad2 pathway is upregulated in patients with RB. ACVR1C ligand Nodal plays an important role in regulating the growth, metastasis, and diffusion of RB. Knockout of the *BANCR* gene can inhibit the proliferation, apoptosis, invasion, and migration of RB cells, and its mechanism may be related to the inhibition of Wnt/β-catenin signaling pathway.[4] However, due to the lack of effective diagnostic methods in the early stage of the disease, the recovery of retinoblasts and the overall survival rate are still not particularly ideal. Therefore, it is very important to clarify the

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The datasets generated during and/or analyzed during the current study are publicly available.

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exact molecular mechanism of occurrence, proliferation, and recurrence of RB, to develop effective diagnosis and treatment strategies.

In the past few decades, microarray technology and bioinformatics analysis have been widely used to screen genetic changes at the genome level, which helps us to identify the differentially expressed gene (DEG) and participate in the occurrence and development of RB. However, the false-positive rate in independent microarray analysis makes it difficult to obtain reliable results. Therefore, in this study, 3 mRNA microarray datasets were downloaded and analyzed from gene expression complex to obtain DEG between normal retinal tissue and retinoblast tissue. Subsequently, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, and protein–protein interaction (PPI) network analysis were used to visualize the relationship between genes to help us understand the molecular mechanism of cancer occurrence and development. In summary, a total of 74 DEGs (Fig. 1A) and 24 HUB genes (Fig. 1B) were identified, which may be candidate biomarkers for RB.

2. Methods

This study has been reviewed by the Ethics Review Committee of Ineye Hospital of Chengdu University of TCM.

2.1. Microarray database

GEO (http://www.ncbi.nlm.nih.gov/geo)[5] is a public genomics database in NCBI, which is used to store high throughput gene expression data, chips, and microarrays. The researchers downloaded the gene expression datasets GSE97508, GSE92987, and GSE24673 from GEO. According to the prescribed mode of operation, the probe is transformed into the corresponding gene symbol. Other relevant datasets were rejected because the sample size was too small, or no samples that met the screening criteria or analysis could not be performed.

2.2. Identification of DEGs

The identification of DEGs using GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r, GEO2R) is an interactive network tool in GEO, which can be used to identify the genetic differences between normal and cancerous cells in RB under experimental conditions. The identified results include, but are not limited to, ID, adj.P.Val, P.Value, t, B, log FC, Gene.Symbol. Among them, the corrected *P* value (adj.P.Val) and Benjamin values were used to identify whether the gene was statistically significant and to limit the balance between false-positives. The identified genes were removed or averaged for further analysis, with a standard of: there are no corresponding gene symbols in the probe set, a collection of genes from >1 probe. To screen the genes with statistical significance in the remaining data, it is necessary to meet the absolute value of log fold change ≥1 and adj.P.Val < .01 at the same time.

2.3. KEGG and GO enrichment analysis of DEGs

Using an online bio information database that integrates biological data and analysis tools: The Database for Annotation, Visualization and Integrated Discovery (DAVID, Http://david.Ncifcrf.gov) (version6.8),[6] a database that stores comprehensive information about the functions of genes and proteins, allows users to extract the biological information they need. KEGG and GO are 2 functional modules, the former is a database, which can extract advanced functions and biological systems, which are generated from large-scale molecular datasets in high-throughput experimental techniques.[7] The latter is an online bioinformatics tool that can annotate genes and analyze their biological processes.[8] The researchers used these 2 tools to analyze the function of DEGs and recorded and counted the data with statistical significance ($P < .05$).

2.4. Building PPI network and module analysis

Use the Search Tool for the Retrieval of Interacting Genes (STRIN, Http://string-db.org) (Version 10.0)[9] online database) to predict possible PPI networks. The mechanism of occurrence or development of the disease is closely related to the interaction between proteins. In this study, using the STRING database to construct a DEG PPI network, the statistically significant combination score should be >0.4. The software Cytoscape (version 3.4.0) is a bioinformatics software, which can be used to visualize the molecular interaction network. It is used to draw the PPI network, and the plug-in MCODE is used to determine the most important module. MCODE (version 1.4.2) is an APP for clustering a given network based on the topology to find densely connected regions.[10] The parameters are set as follows: MCODE score >5, degree cutoff value=2, node score cutoff value=0.2, maximum depth=100, k-score=2. The screened genes were recorded and randomly analyzed by KEGG and GO with DAVID.

2.5. Selection and analysis of HUB gene

The selection criterion of the HUB gene was degrees ≥10. The networks of these genes and their co-expressed genes were analyzed by cBioPortal (http://www.cbioportalorg)[11,12] online platform. The biological process of the HUB gene was analyzed and visualized using the software Cytoscape[13])Bio Networks Gene Oncology Tool (BINGO) (version 3.0.3) plugin. The overall survival and disease-free survival of the HUB gene were analyzed by the Kaplan–Meier curve in cBioPortal.

3. Result

3.1. Screening of DEGs in RB

After eliminating the substandard datasets in the microarray, the DEG was filtered (2528 in GSE97508, 2559 in GSE92987, and 1452 in 24673). As shown in the Venn diagram (Fig. 1A), there are 74 duplicate genes between the 3 datasets, of which 40 are upregulated genes and 34 are downregulated genes.

3.2. KEGG and GO enrichment analysis of DEGs

The biological categories of DEG were simplified by DAVID, and the functions and pathways were analyzed. The changes of (BP) in the biological process of DEG are mainly concentrated in the functional changes of mitotic nuclear division, organelle fission, mitotic cell cycle process, nuclear chromosome segregation, cell cycle process, single-organism organelle organization, DNA metabolic process, cytoskeleton organization, cell proliferation, regulation of cellular component organization, molecules, and the changes of molecular function (MF) are mainly concentrated

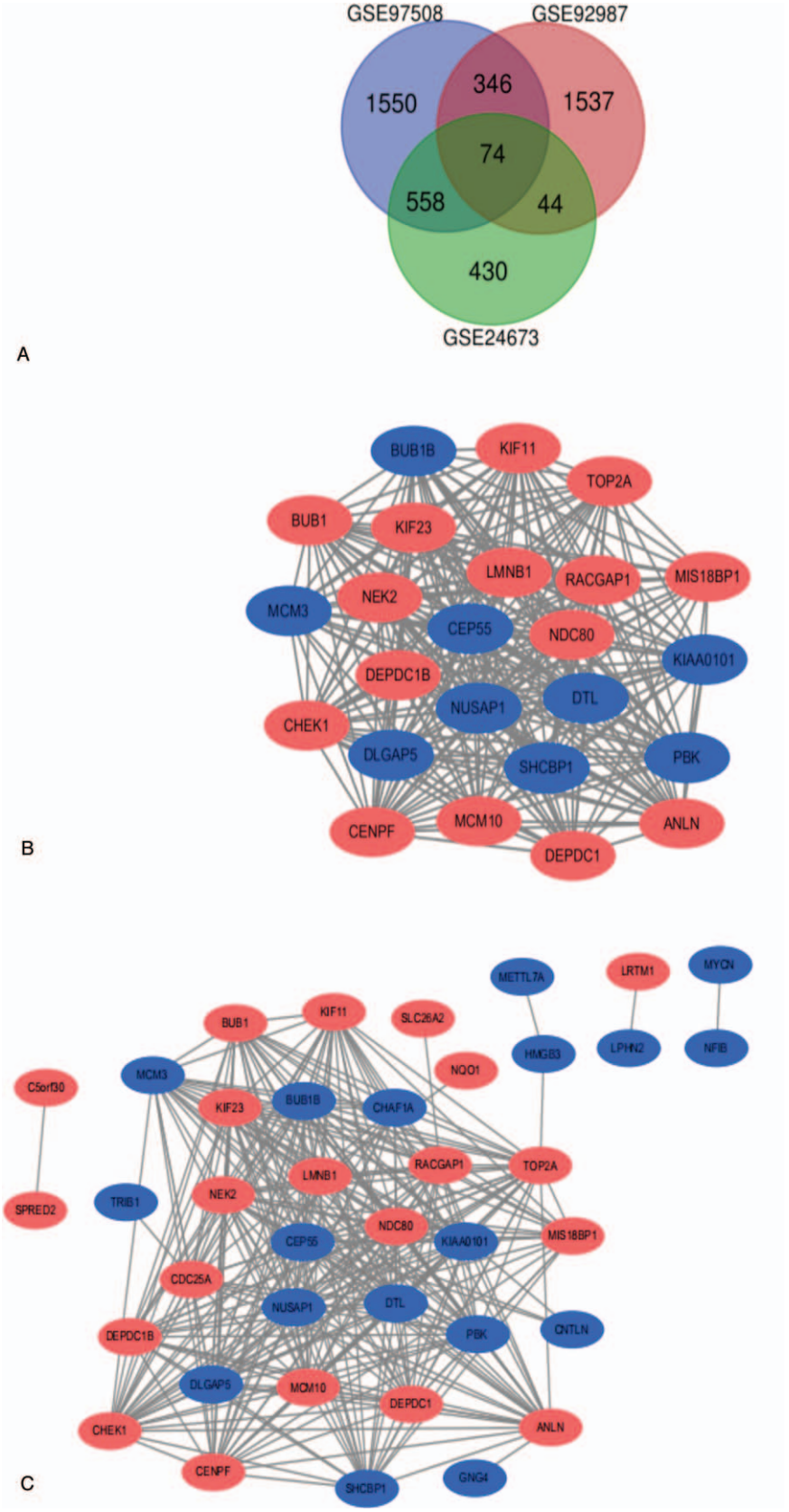

Figure 1. Venn diagram, the most important module of the PPI network and DEGs. (A) Selected DEG with fold change >2 and *P* value <. 01 in mRNA expression profiling sets GSE97508, GSE92987, and GSE24673. The 3 datasets show an overlap of 74 genes. (B) Obtains the most important modules from a PPI network with 24 nodes and 120 edges. (C) Uses Cytoscape to build the PPI network of DEG. The upregulated genes were marked with light red; the downregulated genes were marked with light blue. DEG = differentially expressed genes, PPI = protein–protein interaction

Table 1

GO and KEGG pathway enrichment analysis of DEGs in RB samples.

Term	Description	Count in gene set	*P*
GO:0007067	Mitotic muclear division	16	1.25E-10
GO:0048285	Organelle fission	18	2.56E-10
GO:1903047	Mitotic cell cycle process	20	1.18E-09
GO:0098813	Nuclear chromosome segregation	12	1.80E-08
GO:0022402	Cell cycle pnrocess	22	3.95E-08
GO:1902589	Single-organism organelle organization	17	5.25E-04
GO:0006259	DNA metabolic process	13	5.50E-04
GO:0007010	Cytoskeleton crganization	13	.001849832
GO:0008283	Cell proliferation	15	.014852972
GO:0051128	Regulation of cellular component organization	17	.019933797
GO:0005524	ATP binding	13	.01623841
GO:0032559	Adenyl ribonucleotide binding	13	.019273401
GO:0030554	Adenyl nucleotide binding	13	.020281777
GO:0044877	Macromolecular complex binding	11	.037191448
GO:00420802	Identical protein binding	11	.046594944
hsa04110	Cell cycle	5	2.00E-04

DEGs = differentially expressed genes, GO = Gene Ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, RB = retinoblastoma.

Table 2

GO and KEGG pathway enrichment analysis of DEGs in the most significant module.

Term	Description	Count in gene set	*P*
GO:0005524	ATP binding	9	6.89E-04
GO:0032559	Adenyl ribonucleotide binding	9	8.08E-04
GO:0030554	Adenyl nucleotide binding	9	8.48E-04
GO:0035639	Purine ribonucleoside Triphosphate binding	9	0.002705015
GO:0032550	Purine ribonucleoside binding	9	.002790698
GO:0001883	Purine nucleoside binding	9	.002819737
GO:0032549	Ribonucleoside binding	9	.002819737
GO:0001882	Nucleoside binding	9	.002888434
GO:0032555	Purine ribonucleotide binding	9	.003134187
GO:0017076	Purine nucleotide binding	9	.003274103
GO:0032553	Ribonucleotide binding	9	.003307088
GO:0097367	Carbohydrate derivative binding	9	.009173288
GO:0000166	Nucleotide binding	9	.013408876
GO:1901265	Nucleoside phosphate binding	9	.013441805
GO:0036094	Small molecule binding	9	.020483732
hsa04110	Cell cycle	4	2.26E-05

DEGs = differentially expressed genes, GO = Gene Ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes.

in ATP binding, adenyl ribonucleotide binding, adenyl nucleotide binding, macro mode cult complex binding. The changes of (CC) in DEGs cells were mainly concentrated in the organelle, membrane-enclosed lumen, cell part, cell. KEGG pathway analysis showed that DEGs were mainly enriched in the cell cycle (Table 1).

3.3. Construction and module analysis of PPI network

The PPI network of DEG is built (Fig. 1B) and the most important modules are obtained using Cytoscape (Fig. 1C). DAVID was used to analyze the function of the genes involved in the module. The results show that the genes in this module are mainly enriched in regulating energy metabolism in vivo, promoting the binding of some small molecules and regulating the cell cycle (Table 2).

3.4. Screening and analysis of HUB gene

A total of 24 genes were identified as central genes with degree ≥10. Using the Gene (https://www.ncbi.nlm.nih.gov/gene/) section of NCBI, the full name, abbreviation, and function of

Table 3

Functional roles of 24 HUB genes with degree ≥10.

Number	Gene symbol	Full name	Function
1	ANLN	Anillin actin-binding protein	Encodes an actin-binding protein that plays a role in cell growth and migration, and in cytokinesis.
2	CEP55	Centrosomal protein 55	Mitotic exit and cytokinesis, Recruits PDCD6IP and TSG101 to midbody during cytokinesis.
3	DTL	Denticleless protein homolog	Cell cycle control, DNA damage response and translesion DNA synthesis.
4	MCM3	Minichromosome maintenance complex component 3	Initiation of eukaryotic genome replication
5	MCM10	Minichromosome maintenance 10 replication initiation factor	Initiation of eukaryotic genome replication.
6	CHEK1	Checkpoint kinase 1	Checkpoint mediated cell cycle arrest in response to DNA damage or the presence of unreplicated DNA.
7	BUB1	BUB1 mitotic checkpoint serine/threonine kinase	Encodes a serine/threonine-protein kinase that plays a central role in mitosis.
8	NDC80	NDC80 kinetochore complex component	Encodes a component of the NDC80 kinetochore complex.
9	LMNB1	Lamin B1	Encodes one of the 2 B-type lamin proteins and is a component of the nuclear lamina.
10	BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B	Encodes a kinase involved in spindle checkpoint function.
11	RACGAP1	Rac GTPase activating protein 1	Encodes a GTPase-activating protein (GAP) that is a compoment of the centralspindlin complex.
12	KIF23	Kinesin family member 23	The protein encoded by this gene is a member of kinesin-like protein family.
13	NEK2	NIMA related kinase 2	Encodes a serine/threonine-protein kinase that is involved in mitotic regulation.
14	KIF11	Kinesin family member 11	This gene encodes a motor protein that belongs to the kinesin-like protein family.
15	KIAA0101	KIAA0101	Acts as a regulator of DNA repair during DNA replication.
16	CENPF	Centromere protein F	Encodes a protein that associates with the centromere-kinetochore complex.
17	DEPDC1B	DEP domain containing 1B	Cell migration Source, intracellular signal transduction Source, positive regulation of Wnt signaling pathway, regulation of small GTPase-mediated signal transduction
18	SHCBP1	SHC binding and spindle associated 1	signaling pathways governing cellular Proliferation, cell growth and differentiation.
19	DLGAP5	Disks large-associated protein 5	carcinogenesis of cancer cells.
20	MIS18BP1	Mis18-binding protein 1	Recruitment of CENPAto centromeres and normal chromosome segregation during mitosis.
21	NUSAP1	Nucleolar and spindle associated protein 1	Spindle microtubule organization
22	DEPDC1	DEP domain-containing protein 1A	Transcriptional regulation as a transcriptional corepressor.
23	PBK	PDZ binding kinase	Encodes a serine/threonine protein kinase related to the dual specific mitogen-activated protein kinase kinase (MAPKK) family.
24	TOP2A	DNA topoisomerase II alpha	Encodes a DNA topoisomerase, an enzyme that controls and alters the topologic states of DNA during transcription.

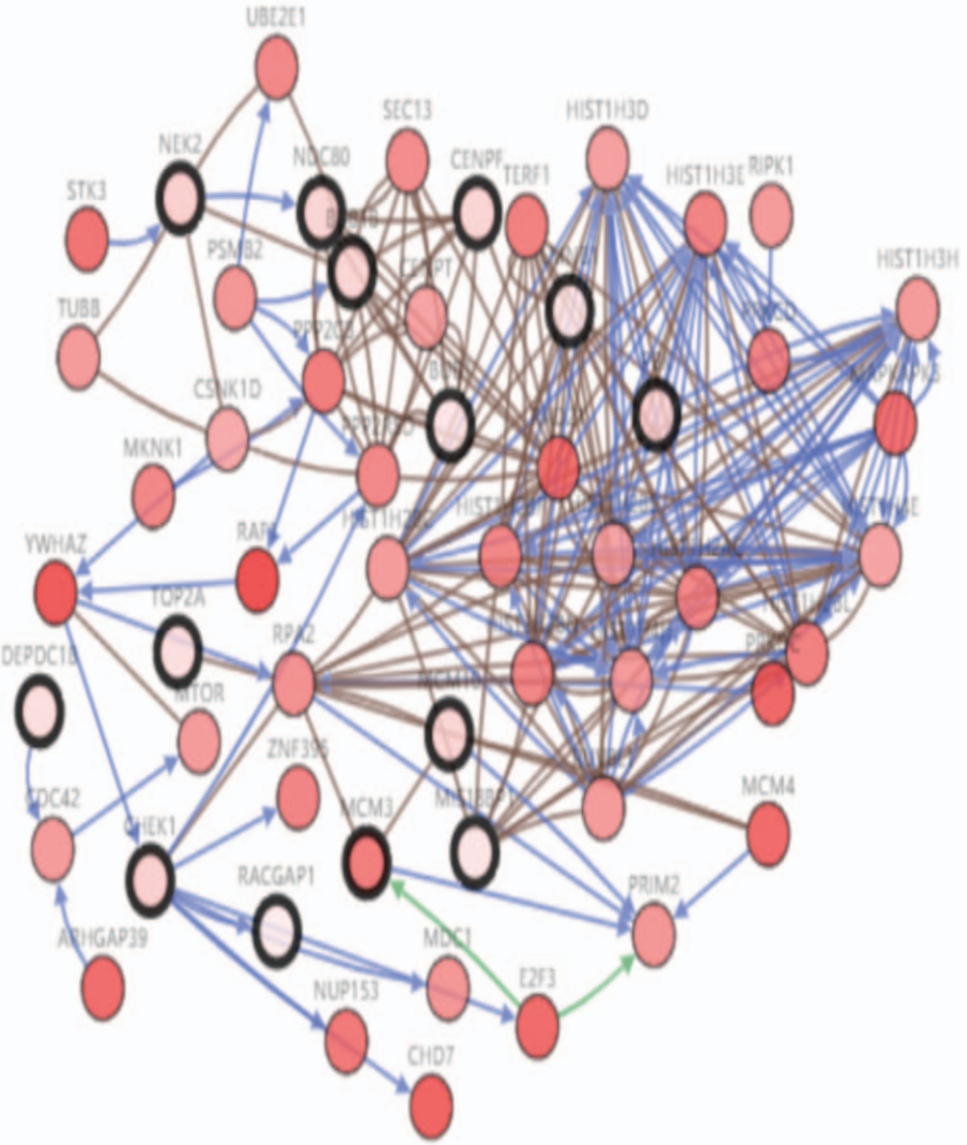

Figure 2. The network of HUB gene and its coexpression gene was analyzed by cBioPortal online platform.

the gene are queried and recorded in Table 3. The network of the HUB gene and its co-expression gene was analyzed by the cBioPortal online platform (Fig. 2). The biological process analysis of the HUB gene is shown in Figure 3. Then, the overall survival of the HUB gene was analyzed by the Kaplan–Meier curve. Among these genes, *PBK* and *LMNB1* have the highest degree of nodes, with 42, indicating that these 2 genes play an important role in the occurrence and development of RB. The overall survival rate was poor in patients with RB with DEPDC1, DLGAP5, SHCBP1, NDC80, MCM10, CEP55 and TOP2A changes (Fig. 4A). At the same time, patients with RB had BUB1B, CENPF, DEPDC1, and DLGAP5 changes that showed poorer disease-free survival (Fig. 4B).

4. Discussion

RB is a common intraocular malignant tumor in pediatrics. The incidence of RB is 116,000[14,15] worldwide. The disease not only seriously damages the visual function of patients, but also endangers the lives of patients. It is reported that RB accounts for about 1%[16] of all cancer deaths between the ages of 0 and 15. The survival rate of RB varies from country to country and ethnicity. The 5-year survival rate of patients in developed countries (such as the United Kingdom and the United States) is 83% to 97%.[17,18] Developing countries such as Africa and India fell to 20% to 48%.[19,20] The earlier it was discovered, the more likely it was to be cured.[15] However, because the visual acuity of children in the early stage of the disease will not be affected, the size of the tumor is small and located in the periphery of the retina, so it is difficult to be detected in time and easy to miss the diagnosis. If the eye symptoms appear after diagnosis and treatment, the possibility of cure is greatly reduced.[21] Therefore, the early detection of RB is very important for the prognosis of patients. It has become a common understanding that gene mutation leads to the occurrence and development of cancer. In RB, studies have shown that abnormal phosphory-

Figure 3. The biological process analysis of HUB gene.

lation of P38 and Hsp27 is involved in the occurrence and migration of RB.[22] CyclinD3, the expression of CDK6 and CyclinE2 in the RB cell line, was abnormally high. Down-regulating the expression levels of *CDK2*, *CDK4*, and *CDK6* genes could significantly increase the apoptosis rate and decrease the migration and invasion ability of the RB cell line in vitro.[23]

However, the study on the molecular mechanism of RB is still not deep enough. There was no successful early screening of RB, which may be one of the reasons for the poor prognosis of the patients. Humans have realized that the treatment of cancer is effective by looking for changes in disease at the molecular level. Therefore, there is an urgent need for potential markers for efficient diagnosis and treatment. Microarray technology enables us to explore genetic changes in diseases and is a useful way to identify new biomarkers in other diseases. In this study, a total of 3mRNA microarray data sets were analyzed to obtain a large number of DEG samples between cancerous and noncancerous tissues. A total of 74 DEGs, were identified, including 40 upregulated genes and 34 downregulated genes. Using different data analysis methods to explore the relationship from various angles, it is found that the rich functions and pathways of DEG include regulating mitosis, cell cycle, DNA transcription process, promoting protein phosphorylation, regulating energy metabolism in vivo, and promote the binding of certain macromolecular complexes to regulate the cell cycle. It is well known that the energy metabolism of the tumor is abnormal, and it has been confirmed that abnormal cell cycle and abnormal mitosis are the most important reasons in the occurrence and development of

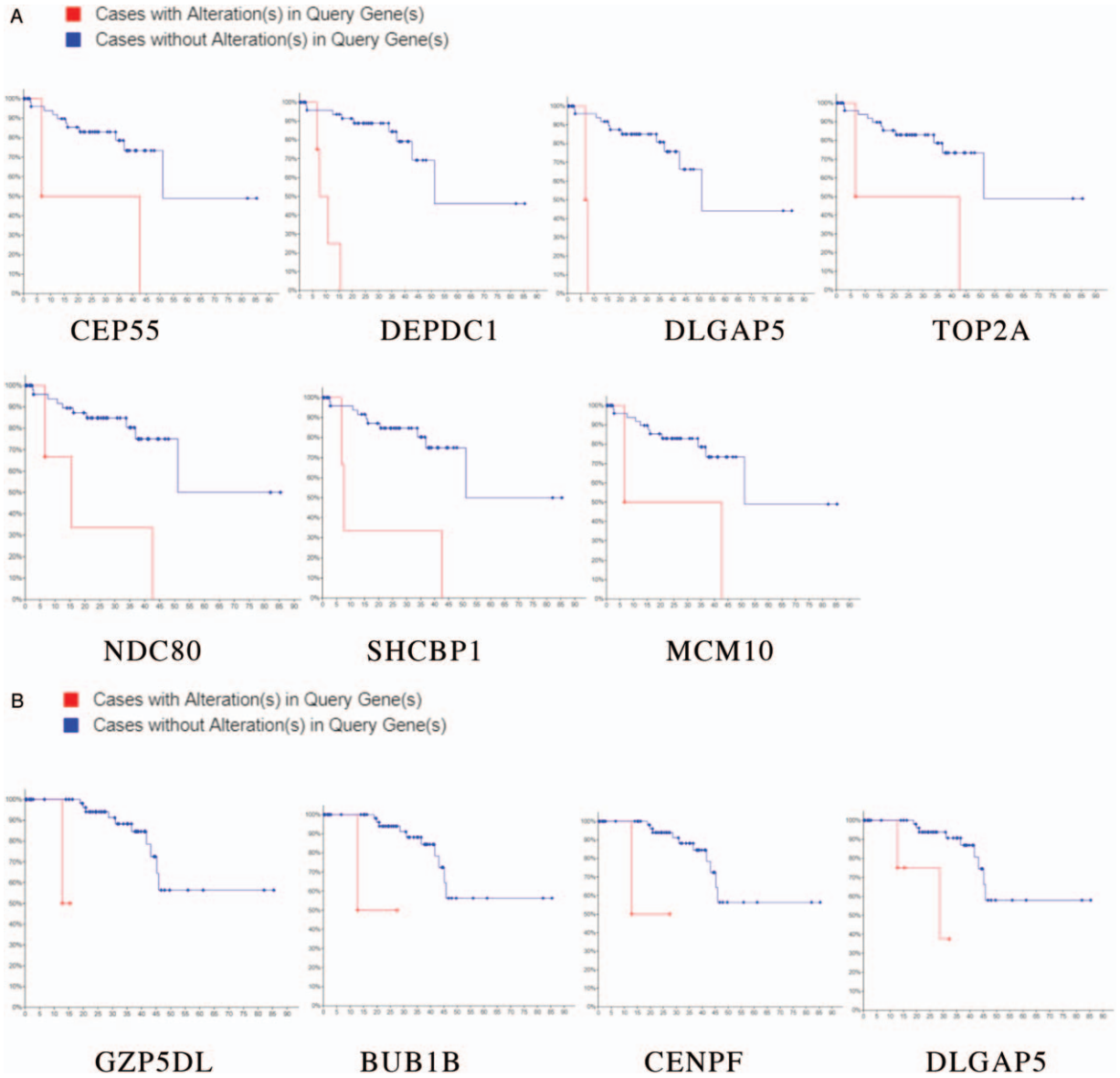

Figure 4. (A) Overall survival and (B) disease-free survival analyses of HUB genes were performed using cBioPortal online platform. $P < .05$ was considered statistically significant. The horizontal axis is the survival time(month), and the vertical axis is the survival state.

tumors.[24–26] On the contrary, studies have proposed complement-activated tumor promotion.[27] Also, oxidoreductase activity usually plays a major role in antioxidant defense and can encode tumor suppressor,[28,29] which is often altered in tumors. In short, all these theories are consistent with our results. GO enrichment analysis showed that the changes of the most significant modules were mainly concentrated in the process of cell proliferation, mitosis, and mitosis, whereas the changes of KEGG were mainly concentrated in the cell cycle. HUB genes are genes that play a vital role in biological processes. In related pathways, the regulation of other genes is often affected by this gene, as is the case in RB. We selected 24 DEGs as HUB genes ≥10 degrees. Among these HUB genes, *PBK* and *LMNB1* had the highest degree of nodes, with 42.

LMNB1 is a membrane protein involved in signal transduction and ion transport,[30] involved in a variety of cell functions, including nuclear stability, chromatin structure regulation, and gene expression.[31] The downregulation of its expression can promote the abnormal gene expression, promote the occurrence of cancer, and play the role of diagnosing cancer and judging the progress of the disease.[6] Knockout of *LMNB1* can slow cell growth and lead to apoptosis.[32] LMNBI is a key protein in RB.[33] Besides, overexpression of LMB1 was observed in prostate, pancreatic, and hepatocellular carcinoma[34–36] and may be considered as a valuable biomarker for tumor diagnosis, treatment, and prognosis.[37]

You can query the PBK function in gene. PBK encodes a serine/threonine-protein kinase related to the dual specific mitogen-activated protein kinase kinase (MAPKK) family. Overexpression of this gene has been implicated in tumorigenesis, and its mechanism may be that alternative splicing results in multiple transcript variants. The relationship between PBK and RB has not been widely reported, but it has been detected in rectal cancer, lung cancer, esophageal squamous cell carcinoma, gastric cancer, and

other malignant tumors. And it is a valuable marker.[38–41] Therefore, we can boldly predict that the abnormal expression of PBK in RB does lead to the occurrence and development of the disease.

In this study, PPI networks showed that PBK and LMNB1 interact directly with most genes, indicating that they are indeed related to the occurrence and development of RB. We evaluated the relationship between the expression of PBK and LMNB1 and overall and disease-free survival. Genetic changes showed a decrease in overall and disease survival. However, in this study, these observations were not statistically significant. We speculate that the reason may be that survival analysis in cBioPortal is based on the relationship between gene mutation and prognosis, and gene overexpression is usually caused by mutation or amplification. Therefore, the overexpression of PBK and LMNB1 in RB may be due to gene amplification rather than mutation, and further research is needed to confirm our hypothesis. At the same time, the results of literature retrieval showed that the interaction between RB and other HUB genes has not been widely reported, but all of them are active in all kinds of malignant cancers. Also, we carried out the hierarchical clustering of HUB genes. The results show that these HUB genes distinguish cancer samples from noncancer samples and may be candidates for diagnostic biomarkers. Also, changes in BUB1, CDC20, KIF20A, RACGAP1, and CEP55 are associated with poorer overall and disease-free survival, suggesting that these genes may play an important role in the occurrence, development, invasion, or recurrence of RB. In conclusion, the purpose of this study was to identify DEGs that may be involved in the occurrence or development of RB. A total of 74 DEGs and 24 HUB genes were identified, which can be used as biomarkers for the diagnosis of RB. Therefore, a more in-depth research is needed to clarify the biological function of these genes in RB.

Author contributions

Conceptualization: Jing Huang, Lingrui Zhang, Zimeng Li.
Data curation: Jing Huang, Lingrui Zhang.
Formal analysis: Jing Huang, Lingrui Zhang.
Software: Jing Huang, Lingrui Zhang.
Supervision: Xuejing Lu.
Validation: Jing Huang, Zimeng Li.
Visualization: Jing Huang.
Writing – original draft: Jing Huang.
Writing – review & editing: Jing Huang, Xuejing Lu.

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