

Identification of microRNA-mRNA regulatory networks and pathways related to retinoblastoma across human and mouse

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Abstract

• **AIM:** To explore the mRNA and pathways related to retinoblastoma (RB) genesis and development.

• **METHODS:** Microarray datasets GSE29683 (human) and GSE29685 (mouse) were downloaded from NCBI GEO database. Homologous genes between the two species were identified using WGCNA, followed by protein-protein interaction (PPI) network construction and gene enrichment analysis. Disease-related miRNAs and pathways were retrieved from miR2Disease database and Comparative Toxicogenomics Database (CTD), respectively.

• **RESULTS:** A total of 352 homologous genes were identified. Two pathways including “cell cycle” and “pathway in cancer” in CTD and enrichment analysis were identified and seven miRNAs (including hsa-miR-373, hsa-miR-34a, hsa-miR-129, hsa-miR-494, hsa-miR-503, hsa-let-7 and hsa-miR-518c) were associated with RB. miRNAs modulate “cell cycle” and “pathway in cancer” pathways via regulating 13 genes (including CCND1, CDC25C, E2F2, CDKN2D and TGFB2).

• **CONCLUSION:** These results suggest that these miRNAs play crucial roles in RB genesis through “cell cycle” and “pathway in cancer” pathways by regulating their targets including CCND1, CDC25C, E2F2 and CDKN2D.

• **KEYWORDS:** Kyoto Encyclopedia of Genes and Genomes pathway; microRNA; retinoblastoma; weighted gene co-expression network analysis

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INTRODUCTION

Retinoblastoma (RB) is a rare malignant retina tumor in children. It initiates during foetal stage and is often delay-diagnosed after birth or during the first few years after birth. The common signs of RB includes leukocoria, strabismus, glaucoma and inflammation^[1-2]. The prognosis and survival of RB has been improved due to the improvement in diagnosis methods and treatment strategies, including chemotherapy regimens, magnetic resonance imaging and surgical therapy^[1-3]. However, the etiology of RB is still not clear till now.

The pathogenesis of RB involves RB tumor suppressor gene biallelic mutation or inactivation and attendant loss of function of RB protein^[1]. It is believed that the molecular features of a disease reflect its origin features and provide clues for treatment. With the development and application of bioinformatics technology, more and more molecular features have been identified to be involved in RB genesis and development. For instance, the knockdown/loss of p107, p130, p53, Cdkn2a and PTEN genes^[4-7], and amplifications of Mdm2 and Mycn^[8-9] benefit to RB development in mouse.

Animal models of cancer, especially mouse models, have attracted researchers' attention long before in cancer field due to the high homologous and identity of human and mouse genome^[10-11]. There are plenty and increasing evidences showing the crucial roles of employing mouse model in identifying key features related to human diseases and cancers, including RB^[10-13]. Researches focusing on the similarity in molecular features of diseases between human and mouse confer the fast development in disease prognosis and treatment^[11]. McEvoy *et al*^[11] identified p53 pathway was suppressed in human RB cells and mouse RB model. It has been widely identified that RB gene is crucial for chromosomal rearrangement and deletion^[14]. RB gene has been identified to be a direct target of various miRNAs^[15-16]. The dysregulation of microRNAs (miRNAs), including miRNA 17-92 cluster, hsa-miR-34a, hsa-miR-373 and hsa-miR-518c are reported to be related with RB development^[17-19]. However, the identity of miRNA-mRNA regulations between human and mouse RB had not been reported till now.

This study was performed to identify the co-expressed miRNA-mRNA regulatory networks and developmental programs in RB between human and mouse. The GSE29683 and GSE29685 datasets were downloaded and homologous genes between human and mouse RB models were identified and analyzed using bioinformatics analyses. Our study would provide new insights into the molecular features in the genesis of RB.

MATERIALS AND METHODS

Microarray Data and Processing Microarray dataset GSE29686 (including GSE29683 and GSE29685) were downloaded from the NCBI GEO database (<https://www.ncbi.nlm.nih.gov/>). GSE29683 and GSE29685 is based on the GPL 570 platform (HG-U133_Plus_2; Affymetrix Human Genome U133 Plus 2.0 Array) and the GPL1261 platform (Mouse430_2; Affymetrix Mouse Genome 430 2.0 Array), respectively. CEL files were processed using the R Oligo software package (v3.4.1; <http://www.bioconductor.org/packages/release/bioc/html/oligo.html>)^[20]. GSE29683 is comprised of 62 RNA samples derived from 55 human RB specimens, 3 passaged xenografts and 4 human RB cell lines (Y79, Weri1, Rb-355, and Rb-13). GSE29685 consisted of 132 primary RB tumor samples from mouse models (SCID mice with *p107*- and *p130*-knockout, *Chx10*^{Cre}, *p53*^{Lox/Lox}, *Rb*^{Lox/Lox}, *MDMX*^{tg}, and *p130*^{Lox/Lox})^[11].

Annotations of Homologous Genes Across Species The homologous genes between human and mouse were converted using the online database mining tool Biomart (v2.3.6; <https://bioconductor.org/packages/release/bioc/html/biomaRt.html>)^[21-22].

Weighted Gene Co-Expression Network Analysis for Cross-Species Genes Weighted gene co-expression network analysis (WGCNA) is a bioinformatics algorithm for the identification of co-expressed network related to disease clinic traits^[23-25]. Different WGCNA modules in GSE29683 and GSE29685 subset were identified using WGCNA package (v1.61; <https://cran.r-project.org/web/packages/WGCNA/index.html>)^[26].

Cross-species comparisons of modules with high topological overlaps (TOs) were performed using fisher's precise test with

enrichment algorithm^[27] as follows:
$$p = 1 - \sum_{i=0}^{x-1} \frac{\binom{M}{i} \binom{N-M}{K-i}}{\binom{N}{K}}$$
,

where N notes total gene number from human samples, M indicates gene counts in each WGCNA module of human, K notes gene number in each WGCNA module of mouse, P value of fisher's precise test is based on the mini number of genes from human (x). TOs with P value <0.05 and fold >1 were defined as significant. The homologous genes between modules with significant TOs were selected as candidate genes associated with RB.

Enrichment Analysis To investigate the biological process and pathways associated with genes, the online software

DAVID (Database for Annotation, Visualization, and Integrated Discovery; v6.8; <https://david.ncifcrf.gov/>) was used. The Gene Ontology (GO) biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with the *P*<0.05 and/or false discovery rate (FDR) <0.05 were defined as significant terms.

Protein-Protein Interaction Network Interactions between the products of homologous genes between human and mouse were identified using Search Tool for the Retrieval of Interacting Genes (STRING, v10.0; <http://string-db.org/>)^[28] with the default setting parameters (reliability threshold 0.4). The protein-protein interaction (PPI) network of homologous genes were visualized using Cytoscape software (v3.6.1; <http://www.cytoscape.org/>)^[29].

Identification and Analysis of RB-Related miRNAs The miR2Disease database (<http://www.mir2disease.org/>) includes various disease-related miRNAs and the detail information of them (including sources, disease, examinations and references)^[30]. miRNAs related to RB were identified from miR2Disease database with the searching term "retinoblastoma".

Construction of miRNA-mRNA and RB-Related Pathway Regulatory Network The targets of miRNAs were predicted in starBase Version 2.0 database (<http://starbase.sysu.edu.cn/>)^[31], and targetScan, picTar, RNA22, PITA and miRanda. The miRNA-mRNA pairs were visualized using Cytoscape. Comparative Toxicogenomics Database (CTD, 2017 update; <http://ctd.mdibl.org/>) was interviewed to identify RB-related KEGG pathways. The searching keyword was "retinoblastoma". The key KEGG pathways and genes related to RB were used to construct RB-related miRNA-mRNA-pathway network.

RESULTS

Identification of Homologous Genes Between Human and Mouse Figure 1A, 1B shows the data processing for GSE29683 and GSE29685, respectively. After normalizing, 13 680 homologous coding genes between human and mouse were identified using Biomart tool. Correlation analysis showed that the 13 680 homologous genes were highly correlated (cor=0.73 and *P*<1e-200) and connected (cor=0.31 and *P*<1e-200; Figure 1C), suggesting the viability of comparative analysis using GSE29683 and GSE29685.

Identification of WGCNA Modules Associated with RB Genesis The 13 680 homologous genes were then used for WGCNA analysis. Figure 2 shows the correlation coefficient square (*r*²) of eigengenes=0.9 and the mean connectivity=1 when the soft threshold power is 10. Accordingly, *r*²=0.9 and soft threshold power=10 were set as the criteria for the identification of WGCNA modules.

Following with the aforementioned criteria combined with cutHeight=0.95 and gene number ≥50, we identified 12 modules (barring grey: HM1-HM14) in GSE29683 (human,

Table 1 Modules and topological overlaps in GSE29683 (human) and GSE29685 (mouse) datasets

Module	Human module	Mouse module	Overlap number	Overlap <i>P</i>	Overlap fold
Black ^a	HM1	MM1	h100(26)m154	3.49E-11	6.124
Blue	HM2	MM2	h404(29)m385	5.49E-02	0.677
Brown ^a	HM3	MM3	h317(69)m332	1.89E-08	2.380
Green	HM4	MM4	h111(3)m195	0.379	0.503
Viridity	HM5	MM5	h45(1)m64	0.556	1.261
Grey	HM6	MM6	h1767(768)m1300	0.000347	1.214
Magenta	HM7	MM7	-	-	-
Pink	HM8	MM8	h71(3)m131	0.744	1.171
Purple	HM9	MM9	-	-	-
Red	HM10	MM10	h102(3)m160	0.802	0.668
Orange	HM11	MM11	-	-	-
Carnation	HM-	MM12	-	-	-
Turquoise ^a	HM13	MM13	h426(257)m470	2.2E-16	4.659
Yellow	HM14	MM14	-	-	-

HM: Weighted gene co-expression network analysis (WGCNA) modules in human GSE29683 dataset; MM: WGCNA modules in mouse GSE29685 dataset. ^aModules with significant topological overlap ($P < 0.05$ and Fold > 1).

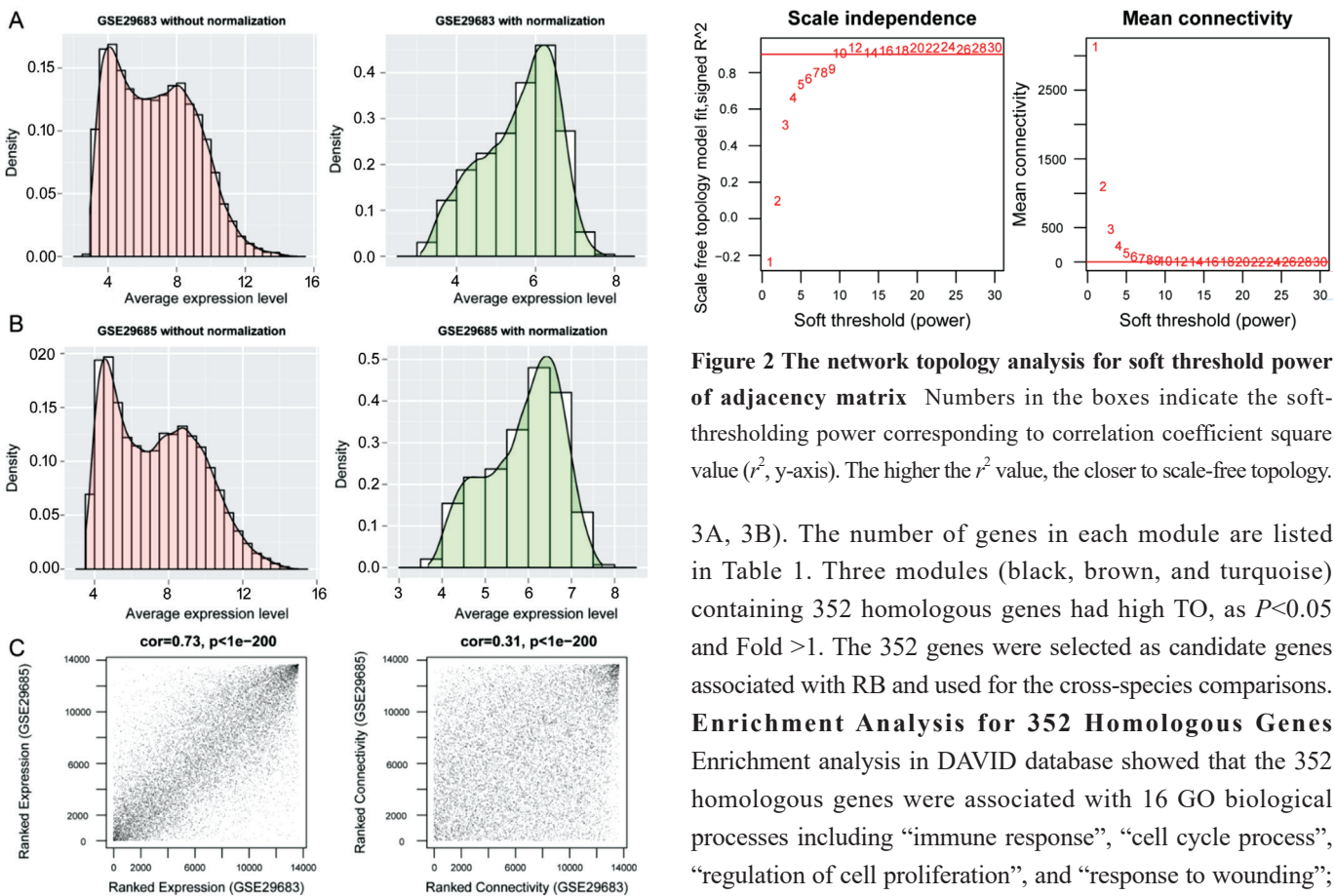


Figure 1 Data normalization and gene correlation in GSE29683 and GSE29685 A, B: The gene density distribution before (left) and after (right) data normalization for GSE29683 and GSE29685, respectively. C: The correlation analysis for expression (left) and co-expression connections of 13 680 homologous genes between the two datasets.

training dataset) and 13 modules (barring grey: MM1-MM14) in GSE29685 (mouse, validation dataset, respectively (Figure

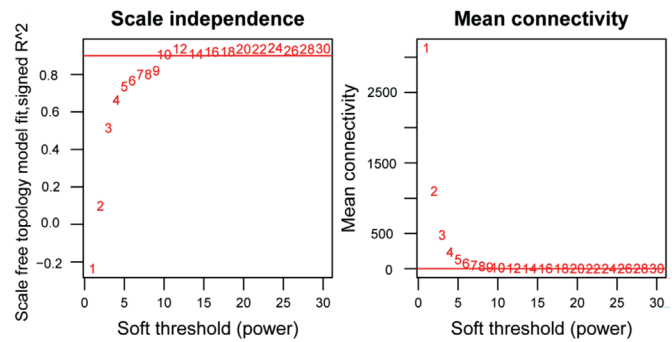


Figure 2 The network topology analysis for soft threshold power of adjacency matrix Numbers in the boxes indicate the soft-thresholding power corresponding to correlation coefficient square value (r^2 , y-axis). The higher the r^2 value, the closer to scale-free topology.

3A, 3B). The number of genes in each module are listed in Table 1. Three modules (black, brown, and turquoise) containing 352 homologous genes had high TO, as $P < 0.05$ and Fold > 1 . The 352 genes were selected as candidate genes associated with RB and used for the cross-species comparisons. **Enrichment Analysis for 352 Homologous Genes** Enrichment analysis in DAVID database showed that the 352 homologous genes were associated with 16 GO biological processes including “immune response”, “cell cycle process”, “regulation of cell proliferation”, and “response to wounding”; and 10 KEGG pathways such as “Toll-like receptor signaling pathway”, “Cell cycle”, “NOD-like receptor signaling pathway”, “Chemokine signaling pathway”, and “B cell receptor signaling pathway” (Table 2).

Protein-Protein Interaction Network Analysis The PPI network based on the 352 genes consisted of 1403 interactions and 251 homologous genes with the threshold of interaction score > 0.6 (Figure 4).

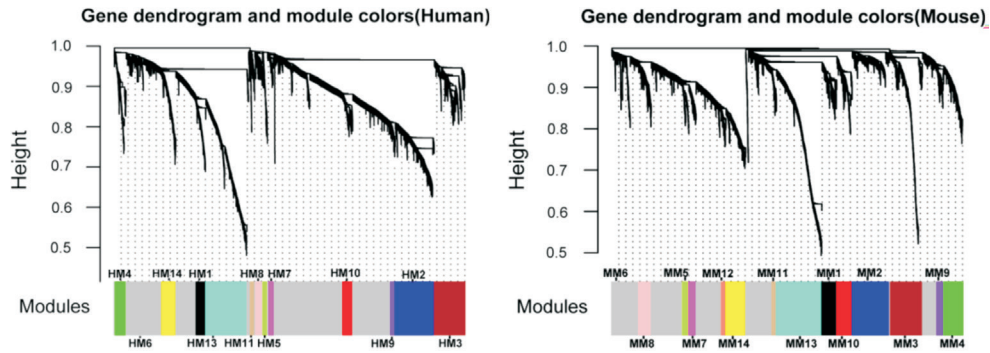


Figure 3 WGCNA analysis for microarray datasets The WGCNA dendrogram for GSE29683 (human) and GSE29685 (mouse), respectively. HM: WGCNA modules in human GSE29683 dataset; MM: WGCNA modules in mouse GSE29685 dataset.

Table 2 GO biological processes and pathways in Kyoto Encyclopedia of Genes and Genomes involved 352 homologous genes between human and mouse

Term	Count	P	FDR
Biological process			
GO:0006955~immune response	55	1.77E-16	3.89E-13
GO:0000279~M phase	38	2.12E-16	3.89E-13
GO:0022403~cell cycle phase	41	2.66E-15	4.60E-12
GO:0007067~mitosis	30	8.18E-15	1.42E-11
GO:0000280~nuclear division	30	8.18E-15	1.42E-11
GO:0000087~M phase of mitotic cell cycle	30	1.32E-14	2.28E-11
GO:0048285~organelle fission	30	2.36E-14	4.06E-11
GO:0007049~cell cycle	55	3.21E-14	5.54E-11
GO:0022402~cell cycle process	46	5.35E-14	9.24E-11
GO:0006952~defense response	48	6.37E-14	1.10E-10
GO:0000278~mitotic cell cycle	36	3.21E-13	5.54E-10
GO:0006954~inflammatory response	33	1.20E-12	2.08E-09
GO:0009611~response to wounding	42	2.06E-12	3.56E-09
GO:0051301~cell division	29	7.98E-11	1.38E-07
GO:0042127~regulation of cell proliferation	43	9.21E-08	1.59E-04
GO:0001775~cell activation	21	6.03E-06	0.010
Pathways			
hsa04610:Complement and coagulation cascades	10	6.55E-05	
hsa04620:Toll-like receptor signaling pathway	11	2.71E-04	
hsa04110:Cell cycle	12	3.79E-04	
hsa04621:NOD-like receptor signaling pathway	7	5.29 E-03	
hsa04062:Chemokine signaling pathway	12	9.34E-03	
hsa04662:B cell receptor signaling pathway	7	1.32 E-02	
hsa04623:Cytosolic DNA-sensing pathway	6	1.39 E-02	
hsa04060:Cytokine-cytokine receptor interaction	14	1.88 E-02	
hsa04670:Leukocyte transendothelial migration	8	3.41 E-02	
hsa04510:Focal adhesion	11	3.68 E-02	

FDR: False discovery rate; GO: Gene Ontology.

MiRNA-mRNA Regulatory Network Thirteen miRNAs were screened in miR2Disease database using searching keyword “retinoblastoma”, including 2 downregulated miRNAs reported by Dalgard *et al*^[18] in 2009 and 11 upregulated miRNAs reported by Zhao *et al*^[19] in 2009 (Table 3). Targets

of the 13 miRNAs were predicted and 107 out of the 352 homologous genes between human and mouse were identified. The corresponding miRNA-mRNA regulatory network was comprised of 186 interactions (lines) and 118 nodes (11 miRNAs and 107 homologous genes, Figure 5).

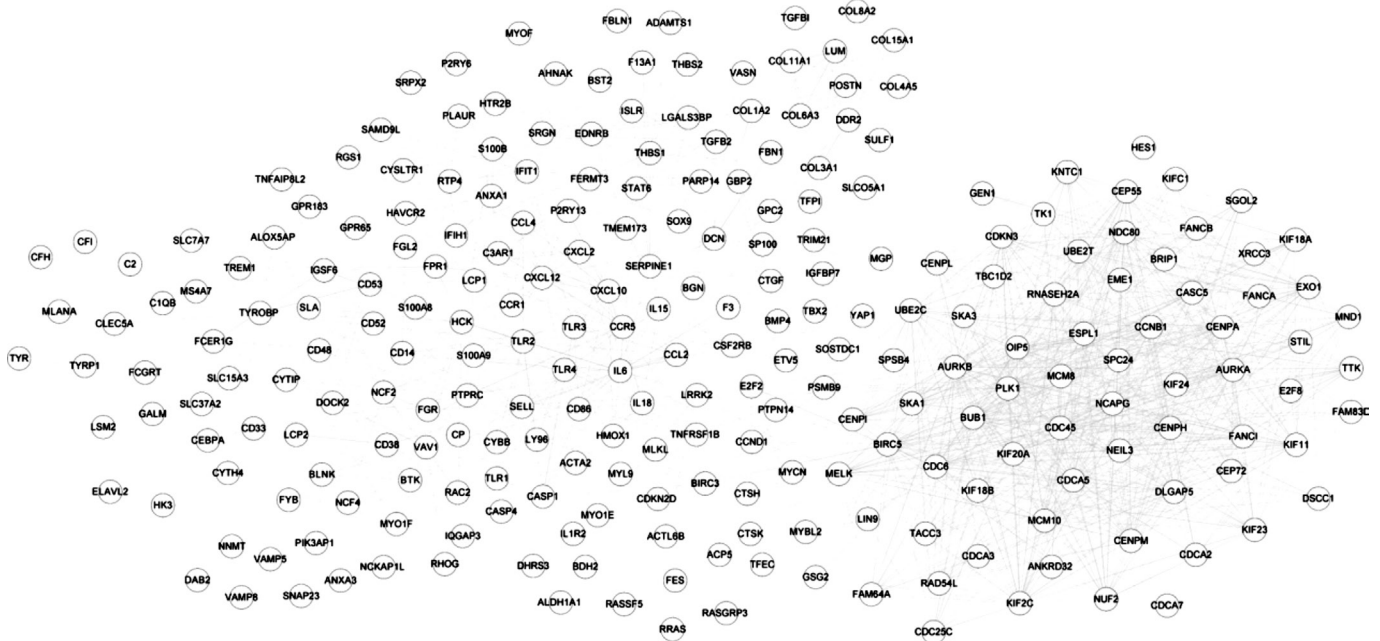


Figure 4 The protein-protein interaction network of the homologous genes between human and mouse Nodes: Products of homologous genes; Lines: Interactions between nodes.

Table 3 Thirteen reported miRNAs that relate to retinoblastoma in miR2Disease database

miRNA	Disease	Regulation	Experiment	Reference
hsa-miR-34a	RB	Down	Northern blot, qRT-PCR <i>etc.</i>	[18]
hsa-miR-34b	RB	Down	Northern blot, qRT-PCR <i>etc.</i>	[18]
hsa-miR-373	RB	Up	Microarray	[19]
hsa-miR-492	RB	Up	Microarray	[19]
hsa-miR-494	RB	Up	Microarray	[19]
hsa-miR-498	RB	Up	Microarray	[19]
hsa-miR-503	RB	Up	Microarray	[19]
hsa-miR-513	RB	Up	Microarray	[19]
hsa-miR-518c	RB	Up	Microarray	[19]
hsa-let-7e	RB	Up	Microarray	[19]
hsa-miR-198	RB	Up	Microarray	[19]
hsa-miR-129	RB	Up	Microarray	[19]
hsa-miR-320	RB	Up	Microarray	[19]

RB: Retinoblastoma.

Enrichment analysis showed these genes in the network were associated with 17 biological processes like “cell cycle phase”, “regulation of cell cycle”, “nuclear division”, “mitotic sister chromatid segregation” and “spindle organization” ($P < 0.05$ and $FDR < 0.05$); and 3 KEGG pathways including “Cell cycle”, “Pathways in cancer”, and “Complement and coagulation cascades” ($P < 0.05$; Table 4).

RB-Related miRNA-mRNA-Pathway Network Sixteen RB-related KEGG pathways were screened from CTD database with keyword “retinoblastoma” (Table 5), including two overlapped KEGG pathways between CTD and DAVID databases (“cell cycle” and “Pathways in cancer”). The miRNA-mRNA-pathway network consisted of 13 genes and 7 miRNAs (Figure 6). Genes including E2F2,

cyclin D1 (CCND1/PRAD-1), CDC25C, CDKN2D, and transforming growth factor- β 2 (TGFB2) gene, baculoviral IAP repeat-containing protein (BIRC5)/surviving gene, bone morphogenetic protein 4 (BMP4) gene, Ras association domain family member 5 (RASSF5) gene /NORE1A, and interleukin-6 (IL-6) gene were involved (Figure 6). Three genes E2F2, CCND1/PRAD-1 and TGFB2 were involved in both pathways. Ten genes were assigned into WGCNA turquoise module and three genes (BMP4, BIRC3 and RASSF5) were in brown module, respectively.

DISCUSSION

The application of WGCNA in biomedicine facilitates monitoring thousands of molecular features associated with disease pathogenesis, development and clinic traits^[32-34]. Using

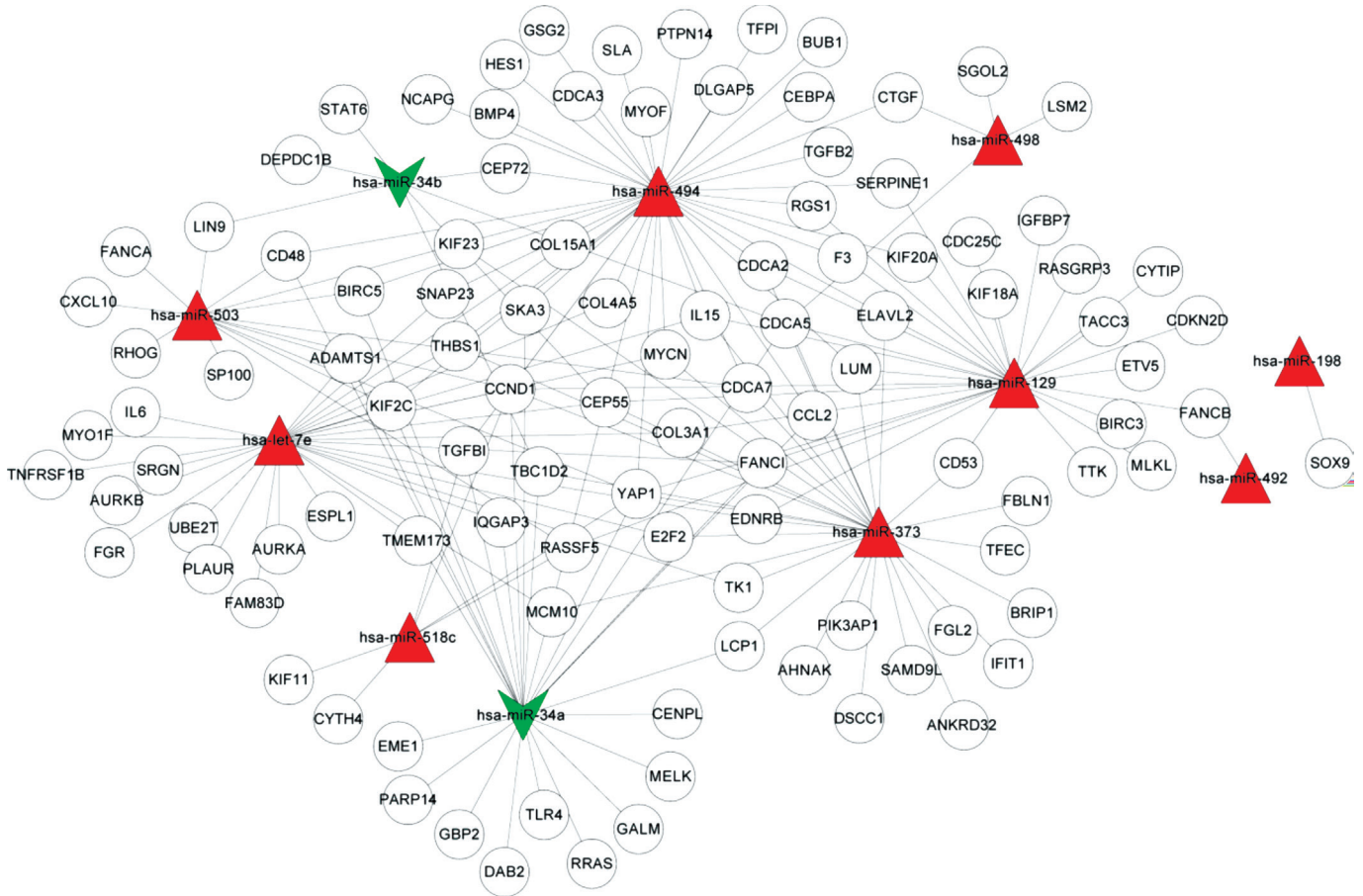


Figure 5 The miRNA-mRNA regulatory network Cycle notes the product of gene. Red triangle and green arrow notes up- and down-regulated miRNA, respectively.

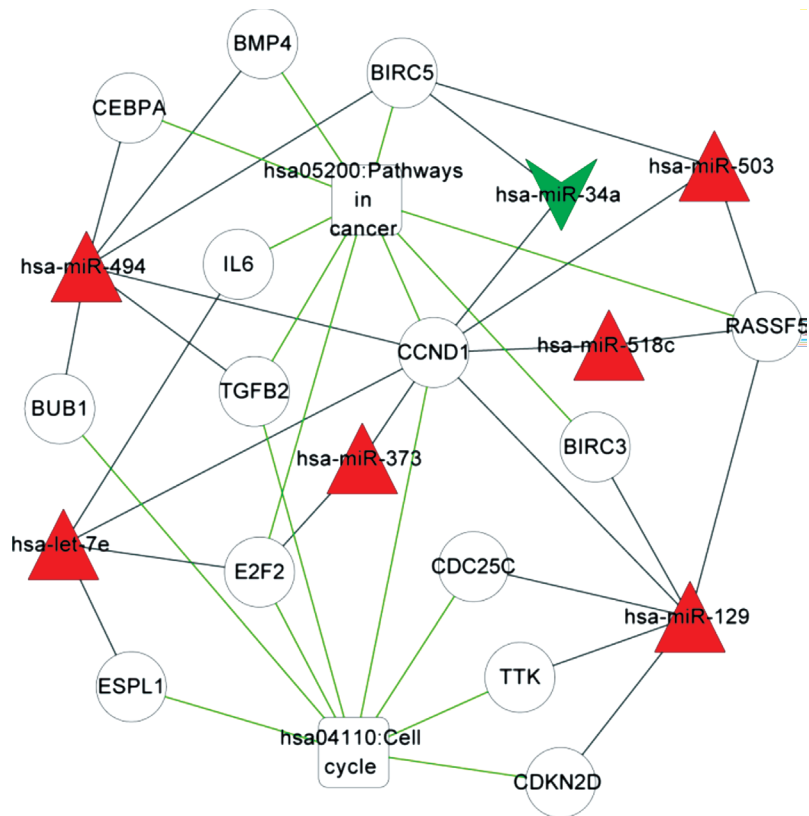


Figure 6 The miRNA-mRNA-KEGG pathway network Box represents KEGG pathway. Cycle nodes the product of overlapped gene. Red triangle and green arrow notes up- and down-regulated miRNA, respectively. Black lines, miRNA-mRNA pairs. Green lines, mRNA-pathway pairs.

Table 4 Biological processes and pathways that involve 107 targets of retinoblastoma-related miRNAs

Term	Count	P	FDR
Biological processes			
GO:0022403~cell cycle phase	25	5.73E-16	9.10E-13
GO:0000279~M phase	23	5.87E-16	9.10E-13
GO:0007049~cell cycle	32	5.92E-16	9.10E-13
GO:0022402~cell cycle process	28	8.66E-16	1.45E-12
GO:0007067~mitosis	19	1.15E-14	1.89E-11
GO:0000280~nuclear division	19	1.15E-14	1.89E-11
GO:0000087~M phase of mitotic cell cycle	19	1.59E-14	2.60E-11
GO:0048285~organelle fission	19	2.34E-14	3.84E-11
GO:0000278~mitotic cell cycle	22	8.22E-14	1.35E-10
GO:0051301~cell division	17	2.04E-10	3.35E-07
GO:0042127~regulation of cell proliferation	23	1.79E-08	2.94E-05
GO:0007059~chromosome segregation	9	9.27E-08	1.52E-04
GO:0051726~regulation of cell cycle	13	3.34E-06	5.48E-03
GO:0000070~mitotic sister chromatid segregation	6	4.87E-06	7.99 E-03
GO:0000819~sister chromatid segregation	6	5.60E-06	9.19 E-03
GO:0000226~microtubule cytoskeleton organization	9	8.79E-06	1.44 E-02
GO:0007051~spindle organization	6	1.50E-05	2.46 E-02
Pathways			
hsa04110:Cell cycle	8	5.14E-05	
hsa05200:Pathways in cancer	9	4.022E-03	
hsa04610:Complement and coagulation cascades	4	1.76 E-02	

GO: Gene ontology; FDR: False discovery rate.

Table 5 Retinoblastoma-related pathways in comparative toxicogenomics database

Disease ID	Pathway	Pathway ID
MESH:D012175	Endocrine resistance	hsa01522
	Cell cycle	hsa04110
	Hepatitis B	hsa05161
	HTLV-I infection	hsa05166
	Epstein-Barr virus infection	hsa05169
	Pathways in cancer	hsa05200
	Viral carcinogenesis	hsa05203
	Pancreatic cancer	hsa05212
	Glioma	hsa05214
	Prostate cancer	hsa05215
	Melanoma	hsa05218
	Bladder cancer	hsa05219
	Chronic myeloid leukemia	hsa05220
	Small cell lung cancer	hsa05222
	Non-small cell lung cancer	hsa05223
	Breast cancer	hsa05224

Bold pathways are overlapped pathways with that in Table 4.

the WGCNA methods, we identified the co-expressed modules and hub genes related to RB genesis in human and mouse. A total of 352 homologous genes between human and mouse were identified as candidates for RB genesis. With WGCNA

and bioinformatics analysis, we found that 10 homologous genes (CCND1, CDC25C, CDKN2D, TGFB2, E2F2, BUB1, CEBPA, TTK, ESPL1 and BIRC5) were clustered in turquoise module, and were involved in “pathways in cancer” and/or “cell cycle” pathways. The other three genes (BMP4, BIRC3 and RASSF5) were clustered in WGCNA brown module and only associated with “pathways in cancer” pathway. These demonstrated that the genes clustered into the same co-expression modules had similar biological functions. The facts that BIRC3, CDC25C and CCKN2D were regulated by hsa-miR-129 and CCND1 was regulated by all 7 miRNAs suggested the crucial roles of these genes and miRNAs in RB genesis and development by interacting “pathways in cancer” and/or “cell cycle” pathways.

RB gene is a nuclear protein in retina cells and is crucial for mitosis, cell cycle, chromosomal rearrangement, and duplications^[14]. Phosphorylated RB protein regulates cell cycle *via* binding to E2F family transcription factors^[35-37]. E2F transcription factor play important roles in cell proliferation and growth in G1 phase by binding with RB protein and S phase by interacting with cyclin A and p¹⁰⁷ protein, which have similar protein-binding properties and structures to RB protein^[36]. CCND1 is an oncogene and its downregulation promotes cell cycle arrest in cancer cells^[38]. CDC25C is a target

of E2F1/E2F2^[39] and CDC25C overexpression suppresses cancer cell proliferation through G2/M phase arrest^[40-41]. Giono *et al*^[41] reported that the suppression of MDM2 gene induced p53 and inhibited CDC25C, respectively. They found MDM2 interacted with CDC25C protein and facilitated CDC25C degradation thus delaying cell cycle *via* arresting cell cycle at G2/M phase. The upregulation of CDC25C in RB tumor tissues has been identified^[42]. Our present study showed that CCND1, CDC25C and CDKN2D were direct targets of hsa-miR-129 and these factors were associated with “cell cycle” pathway. These results suggested that these genes play important roles in RB and might be crucial for the development of RB.

RB gene has reported to be a direct target of several miRNAs including miR-106b and miR-215^[15-16]. Our study showed miRNAs including hsa-miR-373, hsa-miR-503, hsa-miR-129, hsa-miR-518c and hsa-miR-34a were RB-related miRNAs by interviewing miR2Disease database^[18-19]. The broad roles of miRNAs in cancers have been reported by observing the functions of their targets. Elevated hsa-miR-373 and hsa-miR-129 and decreased hsa-miR-34a has been reported in RB tissues in comparison with controls^[18-19,43-44]. Studies had shown that the inhibition of miR-129 was associated with poor outcome of patients with bladder cancer^[45]; elevated miR-129 promoted apoptosis in colorectal cancer (CRC) cells^[46]; hsa-miR-373 was downregulated in CRC tissues^[47], and its expression suppressed gastric cancer metastasis^[48]; miR-34a was downregulated in CRC^[49] and breast cancer stem cells^[50]; the expression of miR-34a blocked breast tumor growth^[50]. We identified 7 miRNAs (including upregulated hsa-let-7e, hsa-miR-373, hsa-miR-494, hsa-miR-503 and hsa-miR-129, and downregulated hsa-miR-518c and hsa-miR-34a) were associated with RB genesis through “cell cycle” and “pathway in cancer” pathways by regulating their targets like CCND1, CDC25C, CDKN2D and E2F2. These results demonstrated the potential roles of these miRNAs in regulating RB genesis and development.

In conclusion, we identified there were 352 homologous genes with high TOs between human and mouse. Bioinformatics analyses showed 7 miRNAs (like hsa-let-7e, hsa-miR-373, hsa-miR-494, hsa-miR-129, and hsa-miR-34a) and 13 genes (including CCND1, CDC25C, CDKN2D, TGFB2 and E2F2) were associated with RB pathogenesis *via* “cell cycle” and “pathway in cancer” pathways. *In vivo* and *in vitro* experimental should be performed to varify these insights.

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