



Identification of four novel prognosis biomarkers and potential therapeutic drugs for human colorectal cancer by bioinformatics analysis

Zhen Sun^{1,2}, Chen Liu¹, Steven Y. Cheng^{1,3,✉}

¹Department of Medical Genetics, ²Department of Pathology and Pathophysiology, ³Jiangsu Key Lab of Cancer Biomarkers, Prevention and Treatment, Collaborative Innovation Center for Cancer Personalized Medicine, Nanjing Medical University, Nanjing, Jiangsu 211166, China.

Abstract

Colorectal cancer (CRC) is one of the most deadly cancers in the world with few reliable biomarkers that have been selected into clinical guidelines for prognosis of CRC patients. In this study, mRNA microarray datasets GSE113513, GSE21510, GSE44076, and GSE32323 were obtained from the Gene Expression Omnibus (GEO) and analyzed with bioinformatics to identify hub genes in CRC development. Differentially expressed genes (DEGs) were analyzed using the GEO2R tool. Gene ontology (GO) and KEGG analyses were performed through the DAVID database. STRING database and Cytoscape software were used to construct a protein-protein interaction (PPI) network and identify key modules and hub genes. Survival analyses of the DEGs were performed on GEPIA database. The Connectivity Map database was used to screen potential drugs. A total of 865 DEGs were identified, including 374 upregulated and 491 downregulated genes. These DEGs were mainly associated with metabolic pathways, pathways in cancer, cell cycle and so on. The PPI network was identified with 863 nodes and 5817 edges. Survival analysis revealed that *HMMR*, *PAICS*, *ETFDH*, and *SCG2* were significantly associated with overall survival of CRC patients. And blebbistatin and sulconazole were identified as candidate drugs. In conclusion, our study found four hub genes involved in CRC, which may provide novel potential biomarkers for CRC prognosis, and two potential candidate drugs for CRC.

Keywords: colorectal cancer, Gene Expression Omnibus, biomarkers, bioinformatics analysis

Introduction

Nowadays, colorectal cancer (CRC) is one of the most deadly cancers and almost 900 000 CRC-related

deaths were reported each year in the world^[1]. With the understanding of pathophysiology of the disease, different treatment options to improve the survival rates of CRC patients have been developed in the

✉ Corresponding author: Steven Y. Cheng, Department of Medical Genetics, Jiangsu Key Lab of Cancer Biomarkers, Prevention and Treatment, Collaborative Innovation Center for Cancer Personalized Medicine, Nanjing Medical University, 101 Longmian Avenue, Nanjing, Jiangsu 211166, China. Tel/Fax: +86-25-86869463, E-mail: sycheng@njmu.edu.cn.

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world. The 5-year survival rate of CRC patients was >90% when the patients were diagnosed at early stages^[2]. However, due to lacking early detection methods, many CRC patients were diagnosed at an advanced stage or in the metastasis status. And the 5-year survival rate for those diagnosed with metastasis was at approximately 12%^[3]. Recently, a new kind of analysis method has been used to identify the differential expression genes between CRC and normal tissues based on the high-throughput sequencing platforms, such as microarrays. This is a promising tool with extensive clinical applications, including molecular diagnosis, prognosis prediction, new drug targets discovery, etc^[4-6]. Furthermore, microarray assay combining bioinformatics analysis made it possible to analyze the gene expression on mRNA level in CRC progression. For example, several studies have used this method to identify key genes in CRC development through comparing with normal samples, and showed that the key genes were involved in different signal pathways, biological processes, and molecular functions^[7-12].

However, with a relatively limited degree of overlap, we still can not find reliable biomarkers or drug targets. Therefore, the discovery of novel biomarkers for early detection and prognosis prediction of CRC is urgently required.

In the present study, we targeted to find key genes to develop novel biomarkers or drug targets for CRC. Therefore, we chose four Gene Expression Omnibus (GEO) datasets, GSE113513, GSE21510, GSE44076, and GSE32323, and used bioinformatics methods to screen the significant differentially expressed genes (DEGs) between CRC tissues and normal tissues. Gene ontology (GO) and KEGG Pathway analyses were used to find the biological roles of these DEGs through DAVID database. Furthermore, the PPI network of DEGs was constructed and key modules or hub genes were selected with Molecular Complex Detection (MCODE) plugin of Cytoscape software. And the clinical significance was validated by GEPIA database. Finally, small active candidate molecules were identified to develop new drugs through Connectivity Map (CMap) database. In brief, we found four hub genes involved in CRC, which may provide novel potential biomarkers for CRC prognosis, and two potential candidate drugs for CRC.

Materials and methods

Data resources

To explore the differential gene expression profiles

between CRC and normal tissues, we searched the NCBI-GEO database to collect enough and adequate tissues. A total of 4 GEO datasets were selected, including GSE113513, GSE21510, GSE44076, and GSE32323. These mRNA profiles were based on platform GPL15207 (GSE113513), GPL570 (GSE21510 and GSE32323), and GPL13667 (GSE44076). A total of 253 CRC samples and 203 normal samples were chosen for this study, including 14 pairs of cancer and normal samples in GSE113513, 124 CRC samples and 24 normal samples in GSE21510, 98 pairs of cancer and normal samples plus 50 healthy donor tissues in GSE44076, and 17 pairs of cancer and normal samples in GSE32323.

Identification of DEGs and data preprocessing

To identify the DEGs, we used the NCBI-GEO2R online tool to analyze these datasets. Subsequently, adjusted P -value <0.05 and $|\log_2(\text{fold change})| > 1$ were set as the cutoff criteria to screen the significant DEGs of each dataset. Finally, Venn diagrams were performed to get the overlap significant DEGs of the 4 datasets.

GO and KEGG pathway analyses of DEGs

To find the biological functional roles of DEGs, GO and KEGG pathway analyses were performed through DAVID database. Significant results of molecular function (MF), biological process (BP), cellular component (CC), and biological pathways were selected with P -value <0.05.

PPI network construction and module analysis

The DEGs profiles were submitted to STRING database for exploring their potential interactions. The interactions with a combined score >0.4 were considered significant. Subsequently, the interaction files were downloaded and imported into Cytoscape software to construct the PPI network. The MCODE plugin was used to find key modules of the whole PPI network with a degree cutoff=2, node score cutoff=0.2, K-core=2, and max depth=100. The hub genes were then selected with connectivity degree >10. Furthermore, KEGG pathway analyses of the significant modules were performed with P -value <0.05.

Analysis and validation of hub genes

To verify the hub genes we found, we used GEPIA database to analyze their expression and clinical

prognostic information in 270 CRC patients. And the survival curve, stage analysis and box plot were performed to show the clinical implications of hub genes.

Identification of small molecules

To find potential small active molecules to develop new drugs for treating CRC, we uploaded DEGs probe profiles into the CMap database. This database can help to predict small molecules that induce or reverse gene expression signature with a score from -1 to 1. And the molecules which value from 0 closer to -1 were functioned as reversing the cancer cell status.

Results

Identification of DEGs in CRC

Analyzed with the GEO2R online tool, a total of 1763, 4411, 2428, and 2276 DEGs were extracted from GSE113513, GSE21510, GSE44076, and GSE32323, respectively, using adjusted *P*-value <0.05 and $|\log_2(\text{fold change})| >1$ as cutoff criteria. The volcano plots of DEGs in each dataset were shown in **Fig. 1A**. And the Venn diagrams showed that 865 overlap DEGs were identified from these four datasets, including 374 significantly upregulated genes and 491 downregulated genes (**Fig. 1B** and **Supplementary Table 1**, available online).

Enrichment analysis of DEGs

To explore the biological functional roles of the overlap DEGs, GO and KEGG analyses were performed on DAVID database. And the top 20 terms were listed in the charts (**Fig. 2A–D** and **Supplementary Table 2**, available online). The GO analysis results consist of three functional categories, including BP, CC, and MF. In the BP group, DEGs were mainly enriched in cell proliferation (**Fig. 2A**). In the CC group, DEGs were enriched in cytoplasm (**Fig. 2B**). And in the MF group, DEGs were enriched in protein binding (**Fig. 2C**). KEGG pathway analysis showed that DEGs were enriched in metabolic pathways, pathways in cancer and cell cycle (**Fig. 2D**). The details of the top 20 terms were listed in **Supplementary Table 2**.

PPI network construction and module analysis

Using the STRING online database and Cytoscape software, a total of 865 DEGs were filtered into the PPI network complex, containing 863 nodes and 5817

edges (**Fig. 3**). Based on degree scores using the MCODE plugin, two key modules were detected from the whole PPI network complex. Module 1 contained 61 nodes and 1648 edges, and DEGs were enriched in cell cycle, oocyte meiosis, progesterone-mediated oocyte maturation, DNA replication and p53 signaling pathway (**Fig. 4A** and **B**). Module 2 had 55 nodes and 625 edges, and these DEGs were enriched in chemokine signaling pathway, ribosome biogenesis in eukaryotes, cytokine-cytokine receptor interaction, pathways in cancer, purine metabolism, RNA polymerase, retrograde endocannabinoid signaling, TNF signaling pathway, legionellosis, regulation of lipolysis in adipocytes, NOD-like receptor signaling pathway, cytosolic DNA-sensing pathway and gastric acid secretion (**Fig. 4C** and **D**). Additionally, the top 20 hub genes, *CDK1*, *CCNB1*, *MYC*, *CCNA2*, *MAD2L1*, *AURKA*, *TOP2A*, *CDC6*, *UBE2C*, *CHEK1*, *RRM2*, *BUB1B*, *TTK*, *TRIP13*, *TPX2*, *BUB1*, *NCAPG*, *KIF2C*, *KIF23*, and *MCM4* were identified with higher degrees of connectivity. These hub genes were enriched in cell cycle, progesterone-mediated oocyte maturation, oocyte meiosis, p53 signaling pathway, and HTLV-I infection (**Fig. 4E** and **F**).

Analysis and validation of hub genes

To validate the hub genes we got from this study, we uploaded the hub genes list into GEPIA database and explored the correlation between hub genes expression and the clinical characteristics of CRC. It was found that *HMMR*, *PAICS*, *ETFDH*, and *SCG2* were significant DEGs in 270 CRC samples from GEPIA (**Fig. 5A**). And these four genes could represent the important prognostic biomarkers for predicting the survival of CRC patients (**Fig. 5B**). Meanwhile, *PAICS* and *SCG2* were related to the stages of CRC progression (**Fig. 5C**). The summaries of four hub genes were shown in **Table 1**.

Identification of related active small molecules

To search candidate small molecules for developing potential drugs to treat CRC, we uploaded DEGs probe profiles into the CMap database. And the predicted results were download and filtered with enrichment score <0 and *P*-value <0.05. The results were shown in **Table 2**. And **Fig. 5D** listed the top 20 small molecules with their enrichment scores and *P*-values. Therefore, these small molecules may be the targets to develop new drugs or therapies of CRC. Among these molecules, Blebbistatin and Sulconazole may be selected for new clinical trials.

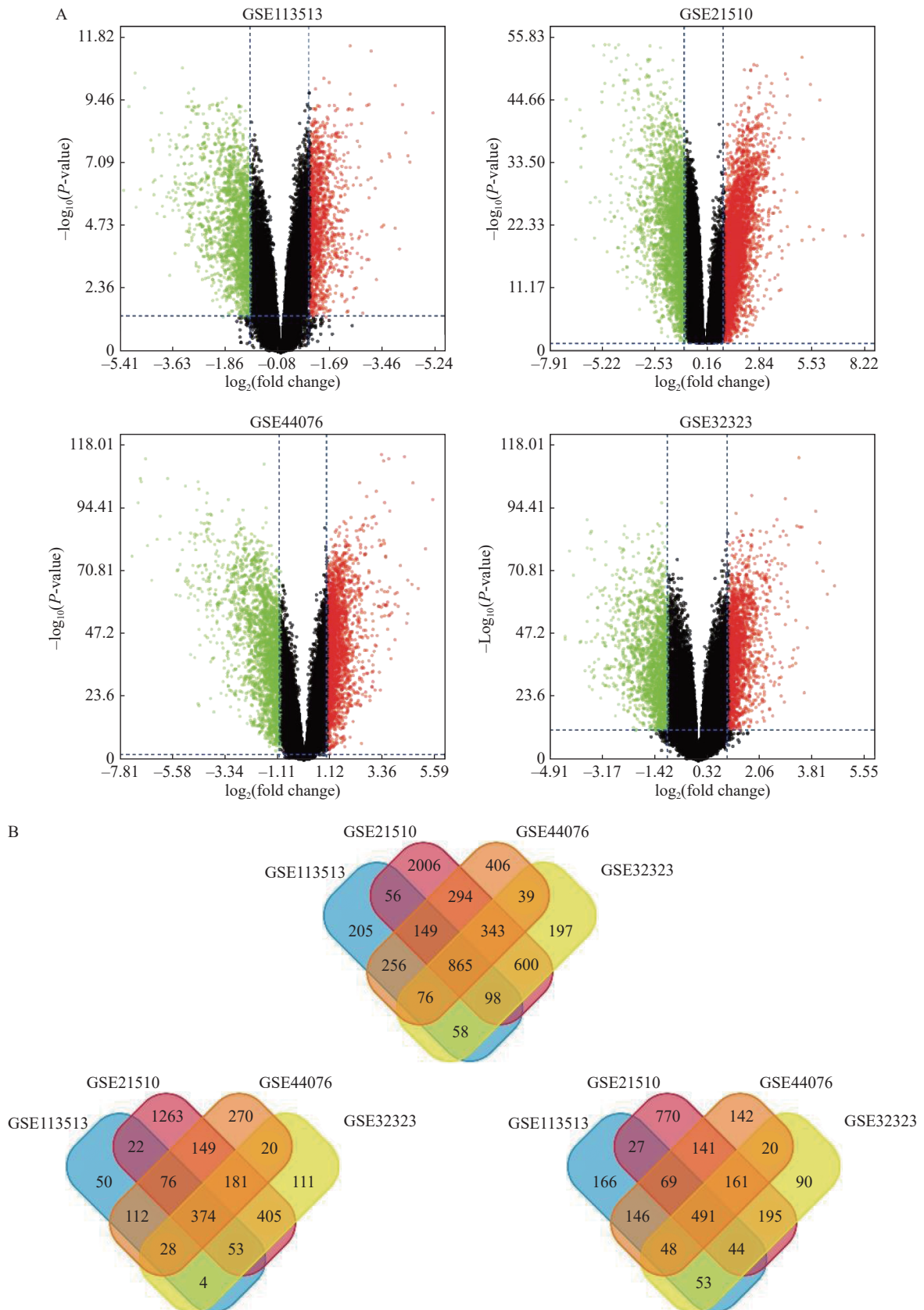
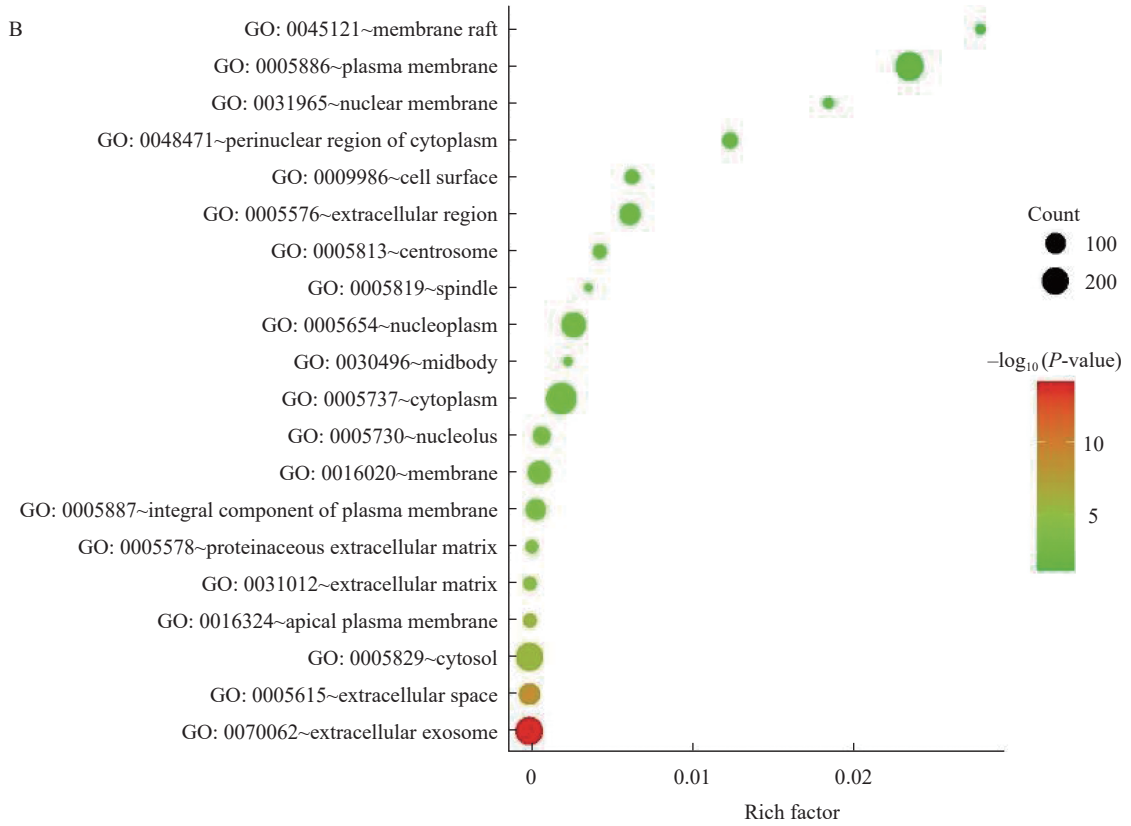
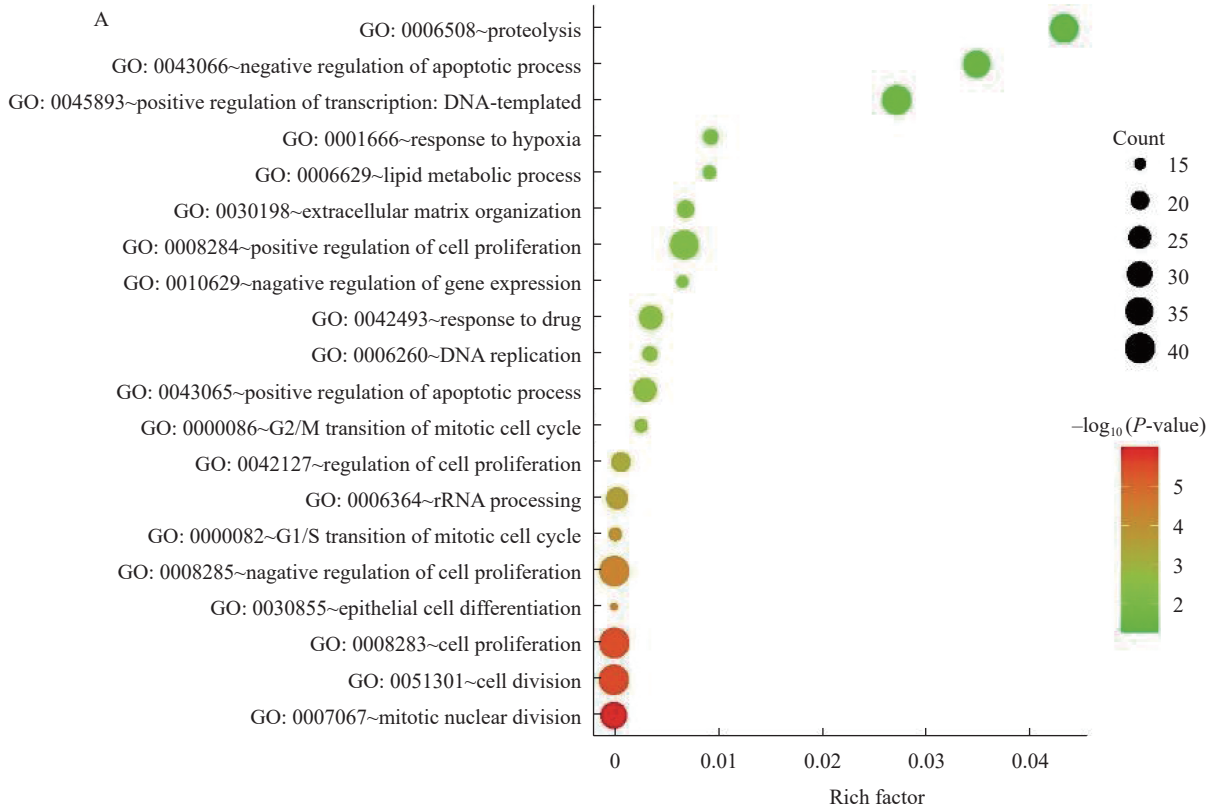


Fig. 1 Identification of the DEGs in CRC. A: Volcano plots of gene expression profiles between CRC and normal samples from GSE113513, GSE21510, GSE44076 and GSE32323. Red dots: significantly upregulated genes in CRC; Green dots: remarkably downregulated genes in CRC. Adjusted P value <0.05 and $|\log_2(\text{fold change})| >1$ were considered as significant criteria. B: Venn diagrams show that 865 overlap DEGs were found through GEO2R in the four datasets, including 374 upregulated DEGs and 491 downregulated DEGs. DEGs: differentially expressed genes; CRC: colorectal cancer.



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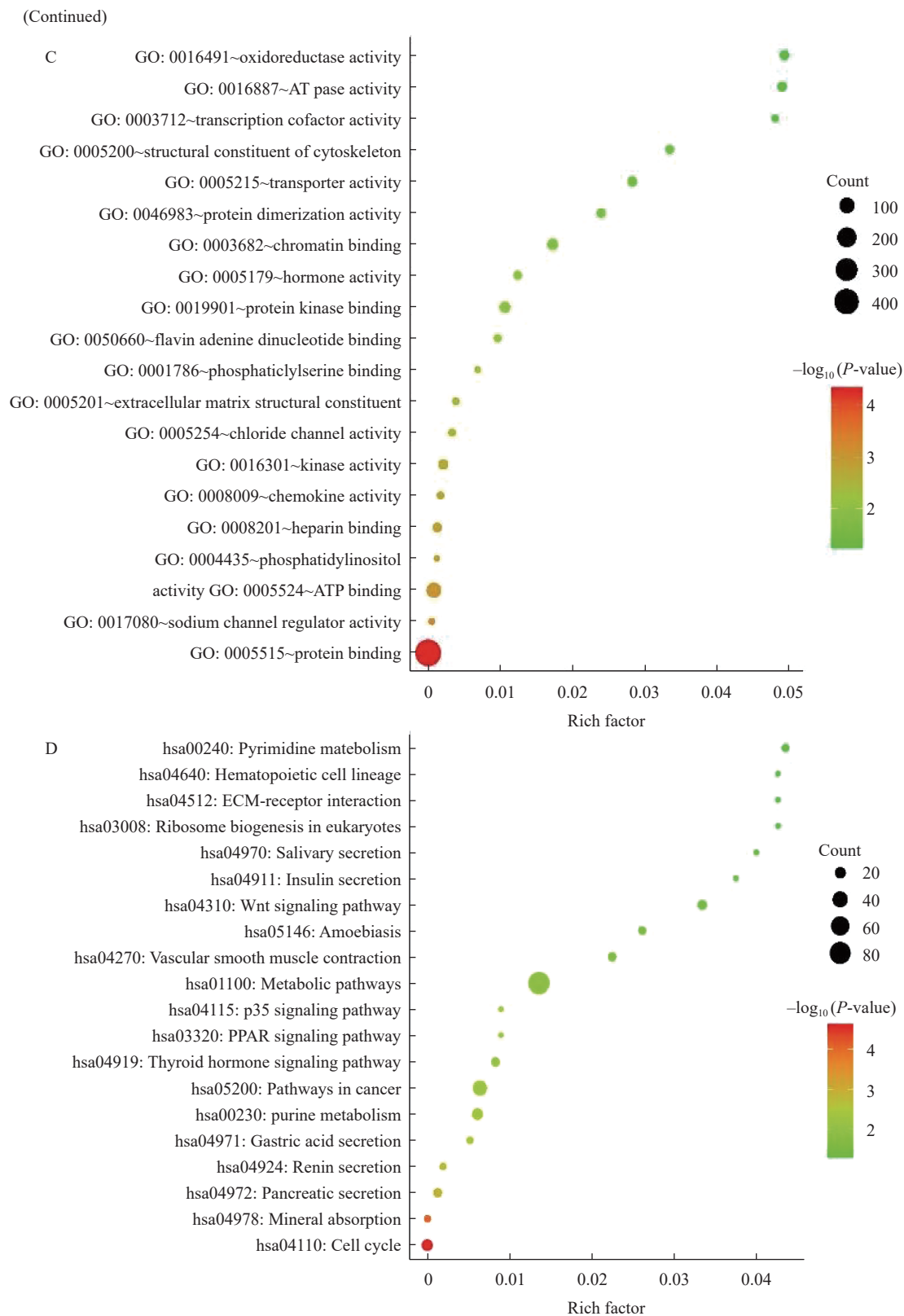


Fig. 2 GO and KEGG analysis of the overlap differentially expressed genes in colorectal cancer through DAVID online-tools. Top 20 terms of biological processes (A), cellular components (B), molecular functions (C), and KEGG signaling pathways (D) were shown in the charts, and P -value < 0.05 was considered as selection criteria.

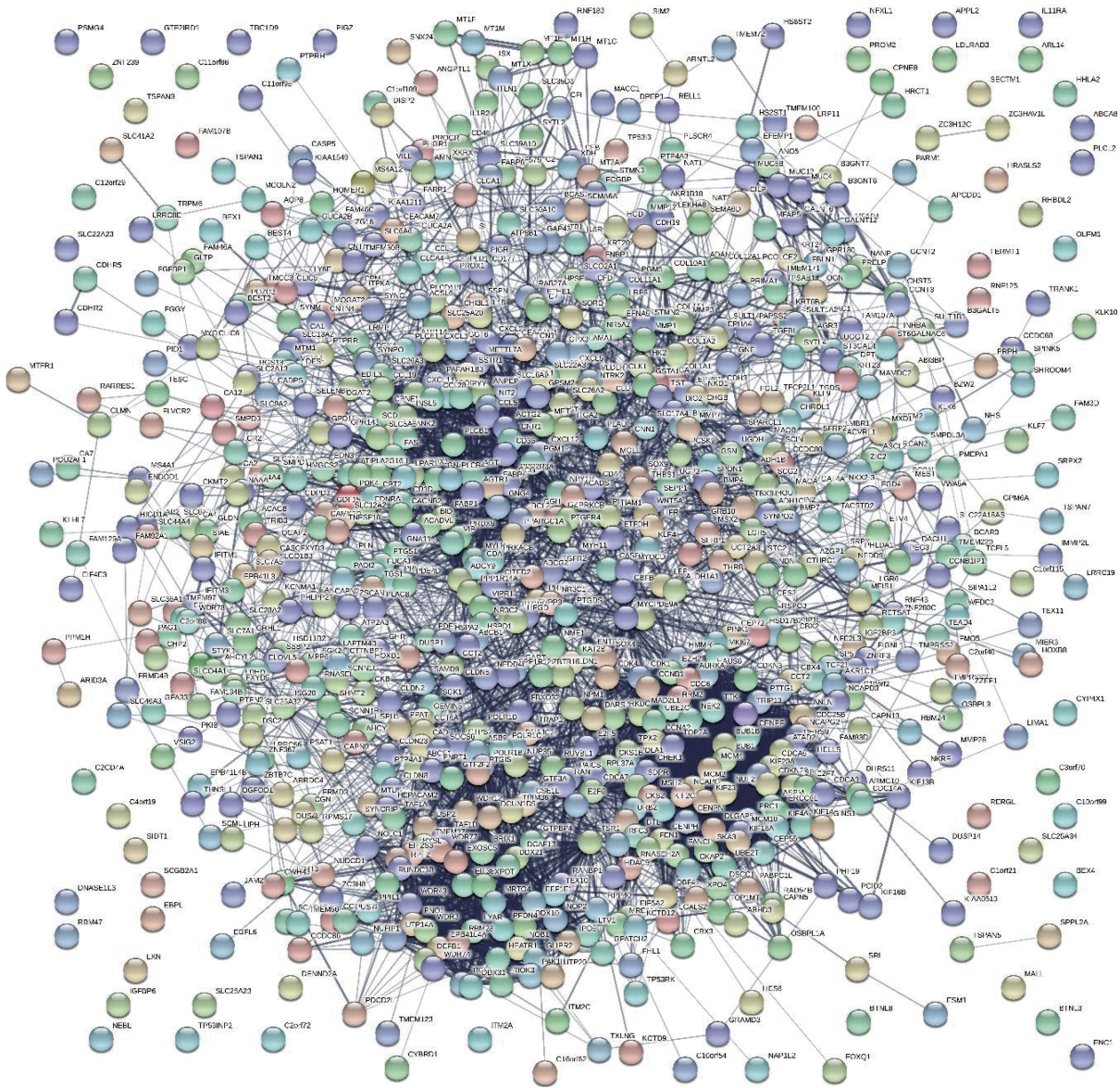


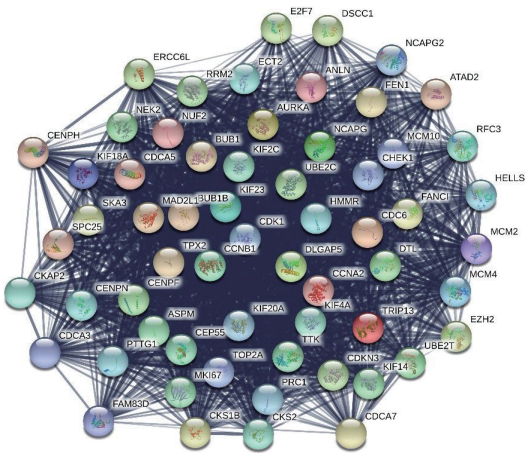
Fig. 3 PPI interaction network of the overlap differentially expressed genes by STRING database.

In summary, we chose GSE113513, GSE21510, GSE44076, and GSE32323 GEO datasets and found 865 significant DEGs between CRC tissues and normal tissues. Subsequently, the biological roles of these DEGs were confirmed with enrichment pathway analysis. Furthermore, the four hub genes, *HMMR*, *PAICS*, *ETFDH*, and *SCG2* were identified as important prognostic biomarkers for predicting the survival of CRC patients based on the GEPIA database. Finally, blebbistatin and sulconazole were picked out to develop new drugs through CMap database (**Fig. 6**).

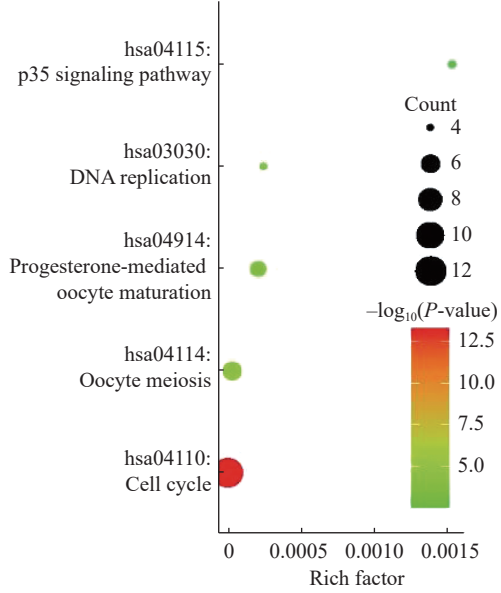
Discussion

In our study, we chose four GEO datasets and used bioinformatics methods to get 865 DEGs (374 upregulated and 491 downregulated). KEGG pathway analysis showed that the key modules were mainly metabolic pathways, pathways in cancer, cell cycle, purine metabolism, pancreatic secretion, thyroid hormone signaling pathway and Wnt signaling pathway. The PPI network was constructed including 863 nodes and 5817 edges. The four hub genes, *HMMR*, *PAICS*, *ETFDH*, and *SCG2* were remarkably

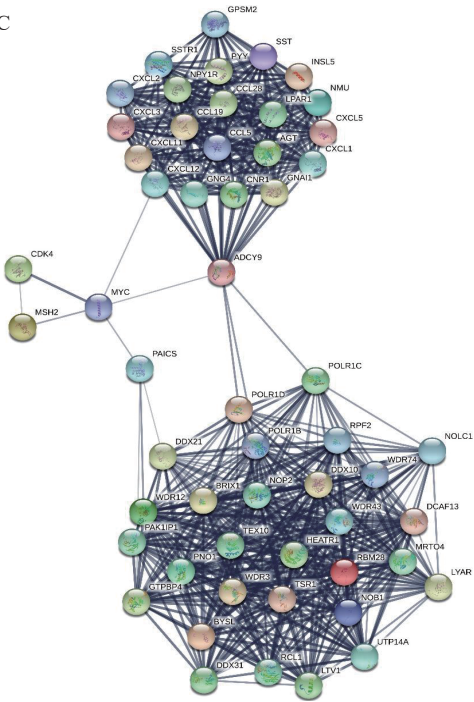
A



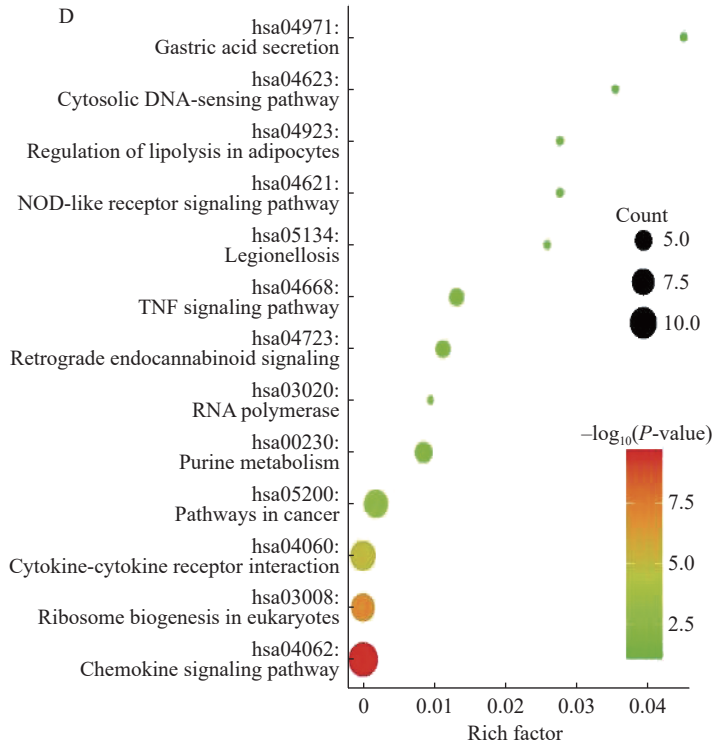
B



C



D



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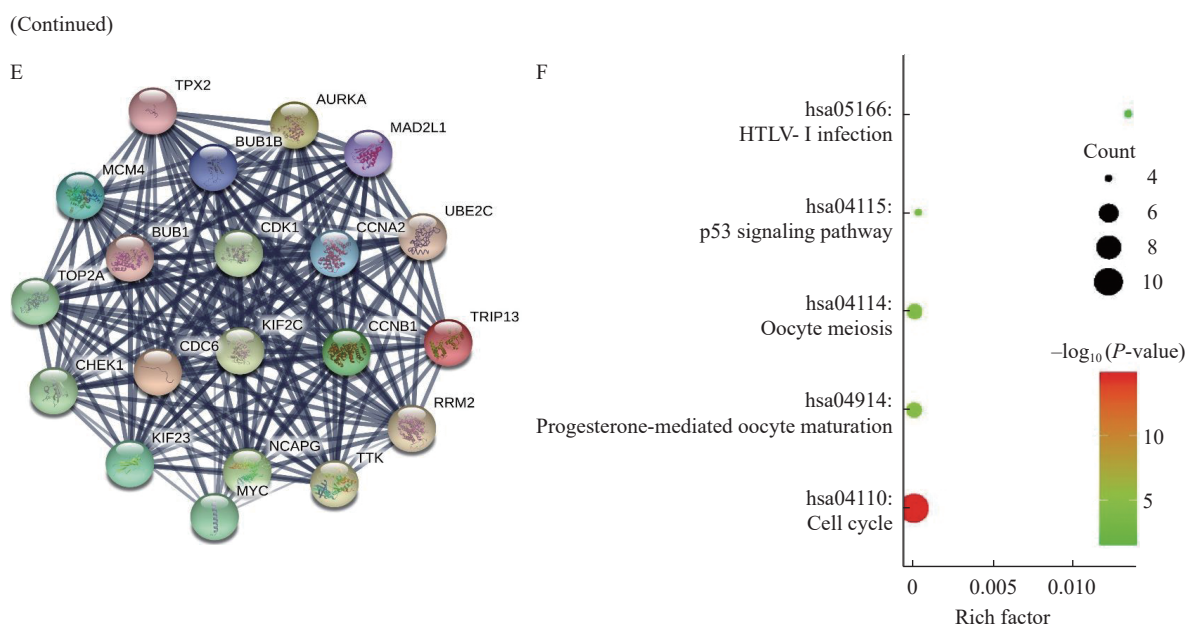


Fig. 4 Module and KEGG analyses of the hub genes. A and C: Two top modules screened from the whole PPI network of DEGs analyzed by MCODE plugin in Cytoscape. E: Top 20 hub genes with a higher degree of connectivity of DEGs were selected with MCODE plugin. B, D, and F: KEGG pathway analysis of the two top modules and top 20 hub genes. DEGs: differentially expressed genes.

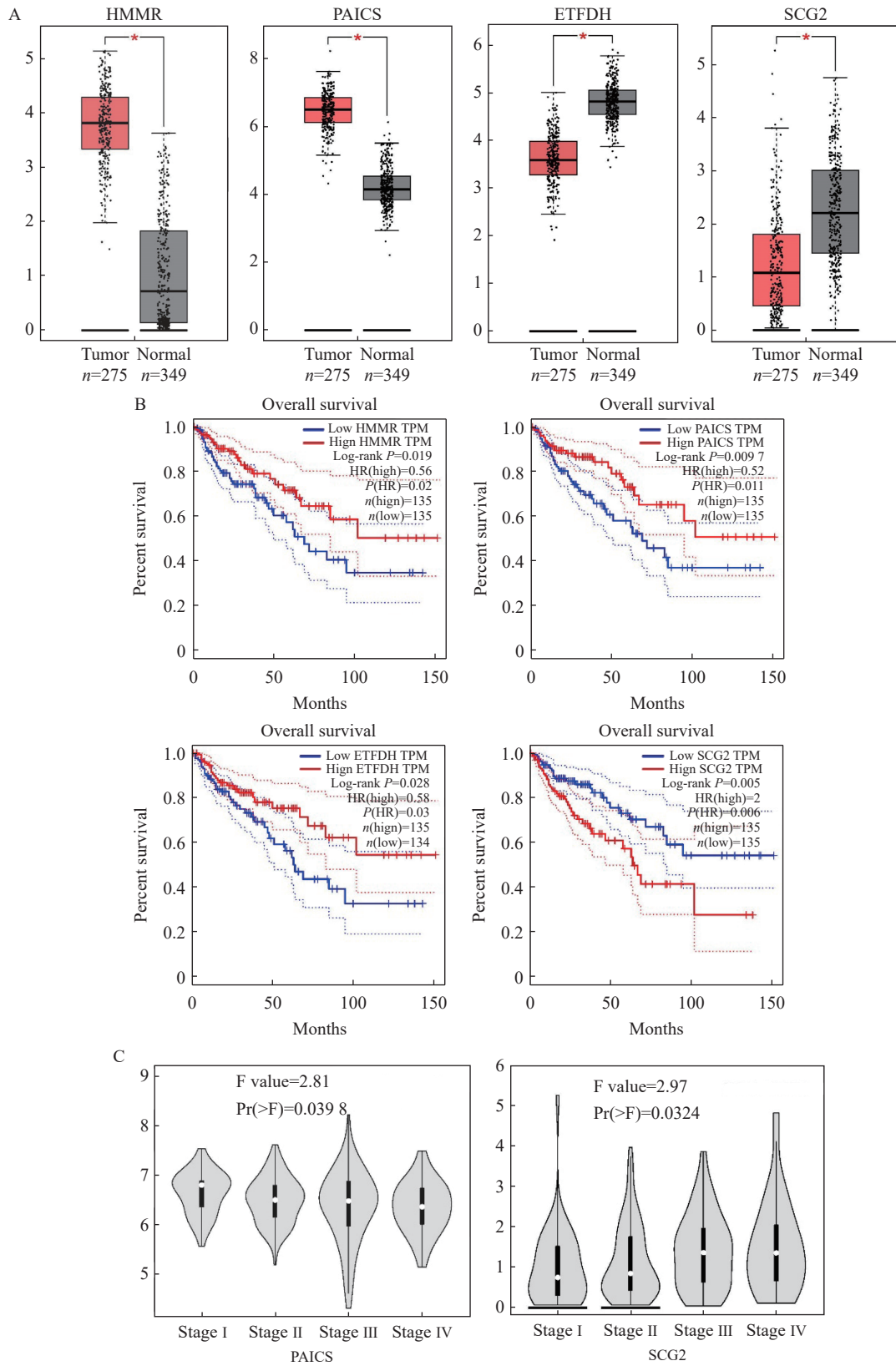
related to the prognosis of patients. Furthermore, two small molecules, blebbistatin and sulconazole, also have been identified as potential candidates to develop new drugs.

Recently, findings about DEGs or molecular biomarkers of CRC have been increasingly reported. Based on integrated analysis of GSE32323, GSE74602, and GSE113513 datasets, and TCGA databases, *CCL19*, *CXCL1*, *CXCL5*, *CXCL11*, *CXCL12*, *GNG4*, *INSL5*, *NMU*, *PYY*, and *SST* were identified as hub genes. And 9 genes including *SLC44A*, *NFE2L3*, *GLDN*, *PCOLCE2*, *TIMP1*, *CCL28*, *SCGB2A1*, *AXIN2*, and *MMP1* was related to predicting overall survivals of CRC patients^[8]. Moreover, *TOP2A*, *MAD2L1*, *CCNB1*, *CHEK1*, *CDC6*, and *UBE2C* were indicated as hub genes, and *TOP2A*, *MAD2L1*, *CDC6*, and *CHEK1* may serve as prognostic biomarkers in CRC^[10]. In addition, *CEACAM7*, *SLC44A*, *GCG*, and *CLCA1* genes were associated with unfavorable prognosis in CRC^[11]. According to analysis of GEO datasets and survival analysis by GEPIA database, *AURKA*, *CCNB1*, *CCNF*, and *EXO1* were significantly associated with longer overall survival. Moreover, CMap predicted that DL-thiorphan, repaglinide, MS-275, and quinostatin have the potential to treat CRC^[9]. In this

study, we have identified four hub genes as new potential biomarkers to predict the prognosis of CRC patients and two new small molecules. Further studies are needed to develop new drugs to treat CRC.

Several studies have reported that these hub genes play important roles in cancer development. For instance, *HMMR* expression level was remarkably correlated with the progression and prognosis of breast cancer^[13], bladder cancer^[14], prostate cancer^[15–16], lung cancer^[17–19], hepatocellular carcinoma (HCC)^[20–22], and gastric cancer^[23]. Furthermore, *HMMR* was confirmed to maintain its oncogenic properties and resistance to chemotherapy through activating TGF- β /Smad-2 signaling pathway^[24]. And *HMMR* was highly expressed in glioblastoma and related to support the self-renewal and tumorigenic potential of glioblastoma stem cells^[25]. *PAICS* was also upregulated in several kinds of cancer tissues and it promotes cancer cells proliferation, migration, and invasion^[26–31]. The expression level of *ETFDH* was found significantly decreased in HCC tissues, and this low expression was related to poor overall survival in patients^[32]. However, the role of *SCG2* in cancer remains unclear.

In the present study, *HMMR*, *PAICS*, *ETFDH*, and *SCG2* were significantly up or down regulated in CRC



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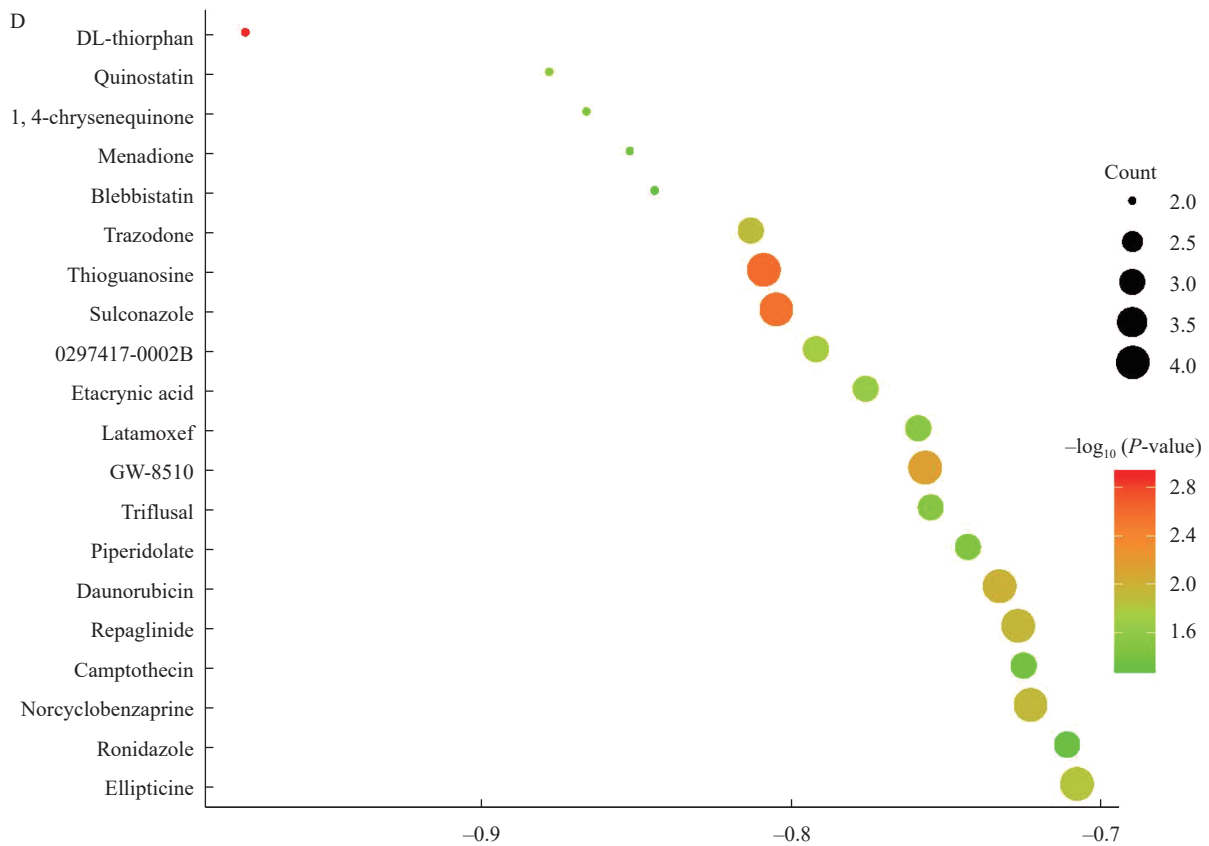


Fig. 5 Analysis and validation of hub genes and identification of related active small molecules. A and B: The expression level and prognostic value of four hub genes based on the GEPIA database. C: PAICS and SCG2 were related with the stages of CRC progression through GEPIA database. D: Top 20 potential small active molecules reverse DEG of CRC predicted by CMap database. TPM: transcripts per million.

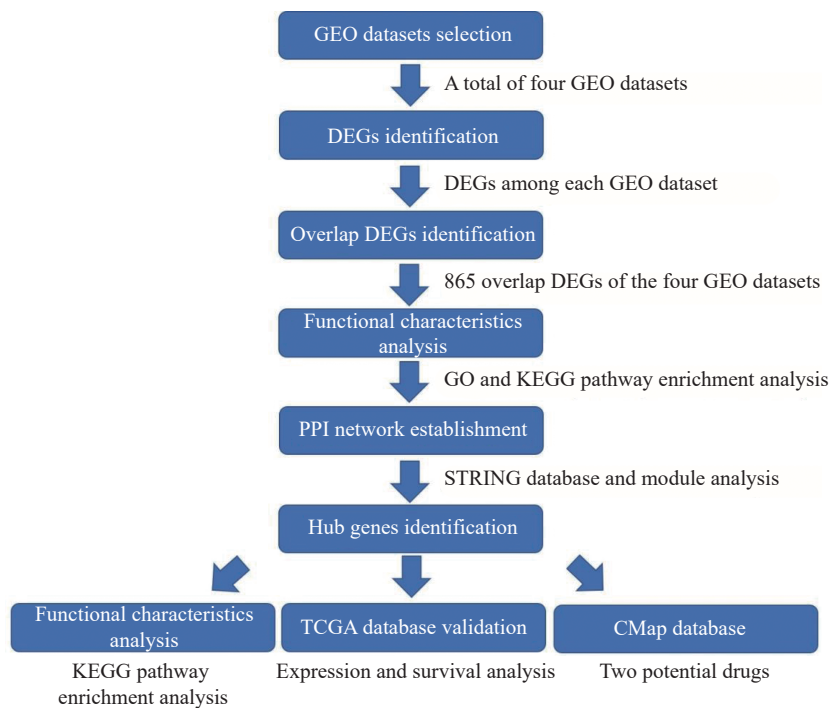


Fig. 6 Workflow model of the present study.

Table 1 Gene summaries of the four hub genes

Gene	Summary	Microarray datasets [<i>P</i> -value, log ₂ (fold change)]			
		GSE113513	GSE21510	GSE44076	GSE32323
<i>HMMR</i>	The protein encoded by this gene is involved in cell motility. It is expressed in breast tissue and together with other proteins, it forms a complex with BRCA1 and BRCA2, thus is potentially associated with higher risk of breast cancer. Alternatively spliced transcript variants encoding different isoforms have been noted for this gene.	6.82e-04, 1.11	3.84e-35, 3.22	5.32e-22, 1.20	2.63e-04, 1.63
<i>PAICS</i>	This gene encodes a bifunctional enzyme containing phosphoribosylaminoimidazole carboxylase activity in its N-terminal region and phosphoribosylaminoimidazole succinocarboxamide synthetase in its C-terminal region. It catalyzes steps 6 and 7 of purine biosynthesis. The gene is closely linked and divergently transcribed with a locus that encodes an enzyme in the same pathway, and transcription of the two genes is coordinately regulated. The human genome contains several pseudogenes of this gene. Multiple transcript variants encoding different isoforms have been found for this gene.	4.77e-08, 1.31	5.71e-47, 2.48	2.14e-55, 1.49	1.38e-06, 1.73
<i>ETFDH</i>	This gene encodes a component of the electron-transfer system in mitochondria and is essential for electron transfer from a number of mitochondrial flavin-containing dehydrogenases to the main respiratory chain. Mutations in this gene are associated with glutaric acidemia. Alternatively spliced transcript variants that encode distinct isoforms have been observed.	1.78e-08, -1.47	1.67e-27, -1.92	1.80e-71, -1.85	3.14e-08, -1.78
<i>SCG2</i>	The protein encoded by this gene is a member of the chromogranin/secretogranin family of neuroendocrine secretory proteins. Studies in rodents suggest that the full-length protein, secretogranin II, is involved in the packaging or sorting of peptide hormones and neuropeptides into secretory vesicles. The full-length protein is cleaved to produce the active peptide secretoneurin, which exerts chemotactic effects on specific cell types, and EM66, whose function is unknown.	2.37e-07, -2.50	1.13e-17, -1.86	4.83e-25, -1.90	2.91e-07, -2.28

tissues compared with those in normal samples, and the survival rate of CRC patients was positively correlated with the expression of these genes. Besides, several small molecules with potential therapeutic efficacy were identified through bioinformatics analyses, including blebbistatin and sulconazole. Blebbistatin has been reported to inhibit cell migration and invasiveness of pancreatic adenocarcinoma^[31], and decrease spreading and migration of breast cancer cells^[33]. Moreover, blebbistatin has shown its antitumorigenic properties in HCC cells^[34]. Another small molecule, sulconazole, also inhibited the

proliferation and formation of breast cancer stem cells through blocking the NF- κ B/IL-8 signaling pathway^[35]. Although these two molecules have significant antitumor activity, their specific roles in CRC development need to be further clarified.

Acknowledgments

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Table 2 The significant small active molecules that may reverse the DEGs of CRC predicted by CMap

CMap name	Count	Enrichment	<i>P</i> -value	CMap name	Count	Enrichment	<i>P</i> -value
DL-thiorphan	2	-0.976	0.001 27	8-azaguanine	4	-0.644	0.038 11
Quinostatin	2	-0.878	0.029 90	Acepromazine	4	-0.640	0.040 08
1,4-chrysenequinone	2	-0.866	0.035 75	Doxazosin	4	-0.639	0.040 48
Menadione	2	-0.852	0.043 24	Mefloquine	5	-0.637	0.016 28
Blebbistatin	2	-0.844	0.048 59	Trifluridine	4	-0.637	0.041 87
trazodone	3	-0.813	0.013 20	Clomipramine	4	-0.636	0.042 51
Thioguanosine	4	-0.809	0.002 59	Trioxysalen	4	-0.633	0.044 24
Sulconazole	4	-0.805	0.002 82	Corbadrine	4	-0.629	0.046 73
0297417-0002B	3	-0.792	0.018 27	Etofenamate	4	-0.624	0.049 27
Etacrynic acid	3	-0.776	0.022 99	Phthalylsulfathiazole	5	-0.616	0.022 71
Latamoxef	3	-0.759	0.028 76	Oxetacaine	5	-0.610	0.025 23
GW-8510	4	-0.757	0.007 12	Amiodarone	5	-0.601	0.029 00
Triflusal	3	-0.755	0.030 11	Meticrane	5	-0.599	0.029 62
Piperidolate	3	-0.743	0.034 63	Vorinostat	12	-0.596	0.000 18
Daunorubicin	4	-0.733	0.010 19	Tranlycypromine	5	-0.592	0.032 64
Repaglinide	4	-0.727	0.011 42	Bromocriptine	5	-0.573	0.042 46
Camptothecin	3	-0.725	0.042 85	Zimeldine	5	-0.572	0.043 38
Norcyclobenzaprine	4	-0.723	0.012 02	Clemizole	5	-0.568	0.045 48
Ronidazole	3	-0.711	0.049 19	Astemizole	5	-0.567	0.046 14
Ellipticine	4	-0.708	0.015 14	Meclozine	5	-0.565	0.047 08
Gliclazide	4	-0.707	0.015 28	Procaine	5	-0.565	0.047 46
Phenoxybenzamine	4	-0.704	0.015 76	Cloperastine	6	-0.551	0.031 56
0175029-0000	6	-0.701	0.001 63	Famprofazone	6	-0.543	0.036 21
Tyloxapol	4	-0.694	0.018 52	Dipyridamole	6	-0.537	0.039 63
Skimmianine	4	-0.690	0.020 01	Promazine	6	-0.523	0.047 85
Bisacodyl	4	-0.690	0.020 03	Trifluoperazine	16	-0.504	0.000 20
Resveratrol	9	-0.684	0.000 04	Prochlorperazine	16	-0.434	0.002 99
Medrysone	6	-0.680	0.002 92	LY-294002	61	-0.428	0.000 00
Bepidil	4	-0.674	0.025 22	Trichostatin A	182	-0.425	0.000 00
Pyrvinium	6	-0.672	0.003 34	Fluphenazine	18	-0.406	0.003 80
Nortriptyline	4	-0.669	0.027 07	Thioridazine	20	-0.388	0.003 27
Apigenin	4	-0.667	0.027 65	Alpha-estradiol	16	-0.357	0.025 35
Methylergometrine	4	-0.664	0.028 71	Geldanamycin	15	-0.349	0.039 26
Bufexamac	4	-0.660	0.030 10	Sirolimus	44	-0.335	0.000 08
Prestwick-1084	4	-0.656	0.032 41	Chlorpromazine	19	-0.329	0.024 17
Deptropine	4	-0.651	0.034 95	Wortmannin	18	-0.314	0.046 91
Dextromethorphan	4	-0.647	0.036 86	Tanespimycin	62	-0.311	0.000 00
Protriptyline	4	-0.646	0.037 36	Fulvestrant	40	-0.225	0.029 99

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