Identification of Hub Gene TIMP1 and Relative ceRNAs Regulatory Network in Colorectal Cancer

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Abstract

Objectives This study aimed to discover the ceRNAs network in pathophysiological development of human colorectal cancer (CRC) and to screen biomarkers for target therapy and prognosis by using integrated bioinformatics analysis.

Methods Data on gene expressions of mRNAs, circRNAs, and miRNAs and clinical information were downloaded from The Cancer Genome Atlas and Gene Expression Omnibus database respectively. Differentially expressed mRNAs (DEGs) were identified by using DESeq2 package of R software. Functional enrichment analysis was conducted by ClusterProfiler package of R software. Protein-protein interaction (PPI) network was shown by STRING website. Survival analysis of hub genes was performed by survival package in R software. Interactions among hub gene, DEmiRNAs and DEcircRNAs were used to construct ceRNAs network.

Results A total of 412 DEGs (including 82 upregulated and 330 downregulated) were screened out between 473 CRC and 41 normal samples. 260 DEcircRNAs (including 253 upregulated and 7 downregulated) were altered. A ceRNAs and PPI network was successfully constructed and TIMP1 associated with prognosis were employed.

Conclusion The present study identifies a novel ceRNAs network, which imply that TIMP1 is a potential biomarker underlying the development of CRC, providing new insights for survival prediction and therapeutic target.

Introduction

Colorectal cancer (CRC) has become a predominant cancer worldwide with more than 1.8 million new cases were diagnosed[1,2]. Annually, more than 900 thousand people died of CRC globally[2,3]. Furthermore, the 5-year survival rates for patients with advanced-stage metastatic cancer are approximately 10%[1]. Like other cancers, CRC is considered as a heterogeneous disease in which gene aberrations, cellular context and environmental influences concur to tumor initiation, progression, and metastasis[4]. Despite the great efforts have been made in the advances in laparoscopic and robotic surgery, more-aggressive resection of metastatic disease, radiotherapy and neoadjuvant and palliative chemotherapies, the new treatments had an insignificant effect on long-term survival[5]. Thus, it is critical to make a thorough inquiry of the underlying biological mechanism of the occurrence and metastasis of CRC associated with prognosis so as to discover novel biomarkers for target therapy and prognosis prediction. Although, accumulating evidence also has demonstrated that multiple genes and cellular pathways participate in the occurrence and development of CRC[6,7]. To date, a lack of knowledge regarding the potential precise molecules and potential mechanisms underlying CRC progression limits the ability to treat this disease.

Bioinformatics analyses, including the analysis of gene interaction networks, microarray expression profiles, and gene annotations, are being utilized as powerful tools for identifying potential diagnostic

and treatment-relevant biomarkers of cancers[8,9]. For example, by analyzing data from the Gene Expression Omnibus (GEO) database, Cao *et al* [10]identified 5 genes (COL1A2, COL1A1, COL4A1, THBS2, and ITGA5) as potential biomarkers and therapeutic targets for gastric cancer. In addition, by analyzing data from GEO and The Cancer Genome Atlas (TCGA), Zhu *et al*. found that high expression of cyclin-dependent kinase 1 (CDK1) is a prognostic factor for hepatocellular carcinoma (HCC), making it a potential therapeutic target and biomarker for the diagnosis of HCC[11]. Particularly, the method of integrated bioinformatics analysis can be used to overcome inaccuracies in sequencing due to small sample sizes. Circular RNAs (circRNAs) are a novel class of endogenous non-coding RNAs that form a covalently closed continuous loop by back-splicing events via exon or intron circularization[12]. Owing to the development of high-throughput sequencing, researchers have discovered that thousands of circRNAs are involved in the progression of oncogenesis, invasion, and metastasis by playing the role of “sponges” to microRNAs (miRNAs)[13]. For instance, Bin[14] verified that circDLGAP4 regulated lung cancer cell biological processes by sponging miRNA-143 to regulate CDK1 expression and circDLGAP4 may serve as a potential biomarker for the diagnosis or treatment of lung cancer. However, at present, most studies involving circRNAs have been limited to the sequencing of a few samples or exploring the biological function of a single circRNAs. To the best of our knowledge, no researchers have yet used integrated analysis of multiple to investigate CRC-related circRNAs.

In this study, differentially expressed genes (DEGs) between human CRC tissues and adjacent non-tumor tissues were identified via analysis of public GEO datasets. Next, to explore the roles of these DEGs, functional enrichment analyses and pathway enrichment analyses were performed. Then, protein-protein interaction (PPI) networks were successfully constructed. The key genes and significant modules in the networks were identified. Kaplan-Meier analysis was performed to evaluate the prognostic value of these hub genes. Furthermore, three additional circRNAs expression profiles were analyzed to identify differentially expressed circRNAs (DEcircRNAs) between CRC and adjacent non-tumor tissues. Finally, DEcircRNA-miRNA-mRNA networks were constructed. The research is expected to help to further elucidate the lncRNA–miRNA–mRNA crosstalk in CRC and generate insight into the potential biomarkers and targets for the diagnosis, prognosis and therapy of CRC.

Materials And Methods

Data collection and preprocessing

Colorectal cancer gene expression profile data was downloaded from TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) and standardized, including 41 normal samples and 473 tumor samples and their clinical information. Colorectal cancer miRNA expression profiles from Illumina HiSeqmiRNASeq platforms, including 8 normal samples and 450 tumor samples, were downloaded from TCGA and standardized. In addition, 4 circRNA expression profiles (GSE121895, GSE126094, GSE138589, GSE142837) from Illumina HiSeqRNASeq platforms, including 23 tumor samples and 23 normal samples, were downloaded from

GEO (http://www.ncbi.nlm.nih.gov/geo) by searching the term “CRC” (July 2020) and errors caused by different chips were removed[15].

Identification of differentially expressed RNAs

The differentially expressed circRNAs (DEcircRNAs), mRNAs (DEGs), and miRNAs (DEmiRNAs) were identified using DESeq2 package of R software (Version 3.5.3; http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html). P value was adjusted to false discovery rate (FDR) and adj.P.Val < 0.05 and $\log_2$fold change (FC)≥2 were set as the cutoff criteria. And R was used to visualize the differential genes. For DEcircRNAs, we use Surrogate Variable Analysis to handle multiple GSE profiles. The volcano maps were plotted based on volcano map of R.

Functional Enrichment Analysis

GO and KEGG analyses are widely used for gene annotation terms and pathway enrichment analysis to identify the biological function of the ceRNAs network. ClusterProfiler package of R software were perform to analyze and visualize functional profiles. P<0.05 was considered as the threshold for significant GO and KEGG terms.

Construction of PPI

The PPI network was conducted to analyze the functional interactions between proteins providing insights into the mechanisms or development of CRC. The minimum required interaction is 0.5. The STRING website (https://string-db.org/) was employed to construct the PPI network.

Prognostic survival assessment of DEGs

According to the median expression level of each DEGs, the CRC patients were divided into high and low expression group. Kaplan-Meier analysis and log-rank test was utilized to paint the survival curves to find the DEGs that were significantly associated with the survival of CRC patients. P value < 0.05 was set as a threshold.

Construction of the ceRNAs network

The DEcircRNAs and DEGs targeted by the DEmiRNAs were retrieved according to starBase[16]. Moreover, the prediction results of TargetScan, miRTarBase and miRDB were integrated by starBase [17–19]. The candidates searched by three databases were intersected with the most important DEmiRNAs. Finally, the DEcircRNA–DEmiRNA–DEGs ceRNAs network was constructed and visualized using the R software.

Quantitative real-time PCR (qRT-PCR) of DEmiRNAs and TIMP1 in CRC and normal tissue

Total RNAs was prepared from colonic tissue using an RNA extraction kit (TIANGEN BIOTECH, Beijing, China) according to the manufacturer's instruction. The extracted RNA was were performed to synthesize cDNA by using FastKing one-step kit (TIANGEN BIOTECH, Beijing, China). qRT-PCR was performed using RealUniversal Color PreMix (SYBR Green) kit (TIANGEN BIOTECH, Beijing, China) to assess the expression of target genes. U6 was used as an internal control for DEmiRNAs. GAPDH was used as internal control for TIMP1. In addition, the relative expression of RNAs was quantified by using the $2^{-\Delta\Delta Cq}$ method.

Results

Identification of DEcircRNAs, DEmiRNAs and DEGs in human CRC

A total of 412 DEGs (including 82 upregulated and 330 downregulated) were screened out between 473 CRC and 41 normal samples with the standard of logFC > 2 and adj.P.Val < 0.05 (Fig. 1A). 260 DEcircRNAs (including 253 upregulated and 7 downregulated) were altered significantly between 23 CRC and 23 normal samples by $\log_2$FC > 1 and adj.P.Val < 0.05 (Fig. 1B). In terms of the DEmiRNAs, 200 DEmiRNAs reached the inclusion criteria in 450 CRC and 8 normal samples including 82 upregulated and 108 downregulated miRNAs (Fig. 1C). The top 10 DEcircRNAs, DEmiRNAs and DEGs are presented in Table 1.

Table 1
Top 10 DEcircRNAs, DEmiRNAs and DEGs in human CRC

	Symbol	Log Fold Change	P value	Type
circRNAs	hsa_circ_0043278	7.35193499	1.42E-07	Up
	hsa_circ_0072088	3.72357337	6.43E-06	Up
	hsa_circ_0006174	6.97297226	2.11E-07	Up
	hsa_circ_0000512	6.69347647	2.83E-07	Up
	hsa_circ_0000518	4.77979328	2.11E-06	Up
	hsa_circ_0000511	4.62226461	2.49E-06	Up
	hsa_circ_0000519	6.75758135	2.64E-07	Up
	hsa_circ_0005273	5.26030064	1.27E-06	Up
	hsa_circ_0000520	6.94732137	2.17E-07	Up
	hsa_circ_0000515	5.22840906	1.32E-06	Up
miRNAs	hsa-mir-374a	7.33005053	2.26E-72	Up
	hsa-mir-486-2	-6.0682766	1.35E-32	Down
	hsa-mir-142	6.04852788	1.06E-29	Up
	hsa-mir-486-1	-6.028201	3.42E-31	Down
	hsa-mir-135b	5.86491219	5.52E-22	Up
	hsa-mir-21	5.80174888	5.68E-114	Up
	hsa-mir-19b-2	5.74040834	1.35E-40	Up
	hsa-mir-19a	5.558011	1.99E-26	Up
	hsa-mir-139	-5.4424734	2.64E-52	Down
	hsa-mir-328	-5.4243365	3.50E-51	Down
mRNAs	AQP8	-6.9967508	3.94E-82	Down
	CA1	-6.4593573	3.89E-111	Down
	GUCA2A	-6.2382921	1.29E-60	Down
	GUCA2B	-6.2006141	3.68E-109	Down
	CLCA4	-6.1925953	1.30E-69	Down
	ZG16	-6.1848044	2.71E-52	Down

	Symbol	Log Fold Change	P value	Type
	SLC26A3	-5.9938727	2.32E-41	Down
	CD177	-5.894624	1.03E-81	Down
	TMIGD1	-5.6845743	6.53E-129	Down
	MS4A12	-5.6317548	1.05E-68	Down

Functional enrichment analysis of GO and KEGG analysis of DEGs

DEGs were functionally classified into biological process (BP), cellular component (CC) and molecular function (MF categories). In the BP category, the top 9 most enriched terms were “regulation of protein processing”, “protein activation cascade”, “regulation of acute inflammatory response” and “complement activation”. In the CC category, the top 4 most enriched terms were “extracellular matrix”, “collagen-containing extracellular matrix”, “blood microparticle” and “apical part of cell”. In the MF category, the 3 most enriched terms were “antigen binding”, “receptor regulator activity” and “receptor ligand activity” (Fig. 2A). In addition, almost 16 KEGG pathways were significantly enriched in our analysis. The top 3 most enriched terms were “cytokine-cytokine receptor interaction”, “Mineral absorption” and “Steroid hormone biosynthesis” (Fig. 2B).

PPI network construction and survival analysis

A total of 412 DEGs (82 upregulated and 330 downregulated) were used to construct the PPI networks, which included 226 nodes and 478 edges. The combined minimum required interaction score□0.5 was considered statistically significant (Fig. 3). 5 genes (CXCL8, TIMP1, CXCL1, SPP1,CXCL12) were confirmed as hub genes and next were taken for survival analysis.

Survival analysis of hub genes

The prognostic value of the 5 hub genes was assessed in CRC patients using Kaplan-Meier analysis and log-rank test. The results indicated that CRC patients with high expression of TIMP1 showed worse overall survival (P = 0.004). In contrast, the other 4 hub genes (CXCL8, CXCL1, SPP1,CXCL12) were not related to the overall survival of CRC patients (p > 0.05) (Fig. 4).

CeRNAs regulatory network construction

Not only CircRNA-miRNA but also miRNA-mRNA interactivity was taken into account, an integrated ceRNAs network was constructed. In order to clearly show the interaction in ceRNAs,the regulatory network contained some well-described biomarkers, for example, hsa-miR-671-5p□hsa-miR-17-3p□hsa-miR-328-3p and TIMP1. The information in this ceRNAs network is particularly crucial for searching the

potential biomarkers for CRC. For instance，hsa-miR-671-5p interacted with TIMP1, while mediated by hsa-circ-0002191. Hsa-miR-17-3p interacted with TIMP1, while mediated by hsa-circ-0023397(Fig. 5).

The expression levels of DEmiRNAs and DEGs in human CRC tissue

To identify the authenticity and feasibility of CeRNAs regulatory network,some vital DEmiRNAs and DEGs are evaluated in CRC and normal tissues. We found that TIMP1 overexpressed in CRC tissue compared to normal tissue (*P＜0.001*). In contrast, the expression level of hsa-miR-671-5p，hsa-miR-17-3p and hsa-miR-328-3p was significantly decreased in CRC tissue (Fig. 6).

Discussion

CRC, the third most commonly diagnosed malignancy and the second leading cause of cancer related death with a notably aggressive biological behavior and a poor survival, has always aroused the close attention for the researchers[2]. It is crucial to identify reliable therapeutic targets and biomarkers in order to improve the clinical outcome for CRC patients. “the ceRNA hypothesis” presents a new pattern of gene expression regulation that cicrRNAs could interact with mRNA through competing with the corresponding miRNA[20]. Subsequently,benefits from the development of sequencing technology and the applications of bioinformatics, increasing biomarkers have been confirmed to play a crucial biological role in the initiation, progression and metastasis of tumor[17–19]. CircRNAs differ from other long non-coding RNAs in the structure, which is characterized with the 5’ and 3’ ends covalently linked. CircRNAs functionally act as miRNAs sponges, RNA-binding protein (RBP) sponges and gene expression regulators, therefore, regulating their target genes expression and proteins network in both of transcriptional or posttranscriptional pattern[21]. Accumulative clinicians consider that circRNAs-miRNAs-mRNAs ceRNA network could provide an integrated view of regulatory crosstalk among these CRC-specific RNA transcripts[22,23].

In this study, DEGs were identified between tumor samples and normal control tissues. Then GO and KEGG analysis were performed to further understand the role of DEGs. The results of GO analysis showed that the DEGs were enriched in regulation of protein processing,protein activation cascade and acute inflammatory which is consistent with the knowledge that protein induced pathology and inflammatory networks underlying CRC is a main cause for tumor development and progression[24–26]. Furthermore，KEGG analysis show that cytokine-cytokine receptor interaction is the key to occurrence of CRC. Cytokines such as TNF-α and IL-6 are classically regarded as central players in CRC by driving activation of the NF-κB and STAT3[27]. Cytokines including IL-11, IL-17A and IL-22 have gained attention as facilitators of CRC[27].

The top degree hub genes (CXCL8, TIMP1, CXCL1, SPP1,CXCL12) was presented in PPI network with DEGs. SPP1, also named Osteopontin (OPN)，has been proven overexpressed in various malignant neoplasms including breast cancer，lung cancer and gastric cancer[28–30]. Although，Kyung-Jin Seo[31] evaluated the expression of SPP1 in 174 stage II and III CRC specimens and found SPP1 is significantly

associated with cell invasion and adherence in CRC，the underlying mechanism was not revealed. Jun-qing Wang [32] has proven that SPP1 functions as an enhancer of cell growth in hepatocellular carcinoma (HCC) targeted by miR-181c. Further study showed that SPP1 promoted the metastasis of CRC by activating epithelial-mesenchymal transition (EMT) [33]. CXCL8 as a prototypical chemokine is responsible for the recruitment and activation of neutrophils and granulocytes to the site of inflammation which demonstrate a crucial role in facilitating tumor growth and progression in breast cancer, prostate cancer, lung cancer, colorectal carcinoma and melanoma[34]. phosphorylation of Src-kinases and FAK in cancer cells were increased in CXCL8 signaling，which contributes to cell proliferation and chemoresistance[35,36]. The levels of CXCL1 are elevated in CRC and increased levels are associated with tumor size, advancing stage and patient survival[37,38]. It was reported that CXCL1 could promote tumor growth by inducing angiogenesis and recruiting neutrophils into the tumor associated microenvironment[39,40]. CXCL1, most abundant secreted chemokine by tumor-associated macrophages (TAMs) have been implicated in the promotion of breast cancer growth and metastasis via activating NF-κB/SOX4 signaling[41]. The similar phenomenon has been observed in human bladder cancer[42]. Some researchers indicated that CXCL1 could increases oncogene expression in colon cancer, including PTHLH, FOXO1, TCF4 and ZNF880 in CXCL1-treated SW620 cells according to transcriptome analysis[43]. Besides that, CXCL1 is vital for premetastatic niche formation and metastasis in CRC[44]. CXCL12 also known SDF-1 is widely distributed in human tissues and more than 23 different types of cancers[45]. Importantly, it has been found that C-X-C chemokine receptor type 4 (CXCR4) and its ligand CXCL12 are implicated in cell proliferation, angiogenesis， migration, EMT and tumor metastasis[46]. TIMP1 belongs to the tissue inhibitor of metalloproteinases family which included TIMP1, TIMP2, TIMP3 and TIMP4[47]. In the present study, TIMP1 has been reported to indicate poor prognosis in CRC (P = 0.004) which is consistent with the research of Guohe Song[48]. He considers that expression of TIMP1 was obviously associated with the regional lymph node and distant metastasis. In addition, TIMP1 was an independent prognostic indicator for the progression and metastasis of colon cancer through FAK-PI3K/AKT and MAPK pathway[48]. The TIMP-miRNA axis has been believed to be a potential therapeutic target against aggressive or drug-resistant for kinds of human cancers. For instance，The angiogenesis and tumor growth were increased when TIMP1 banded to CD63 and stimulated miR-210 accumulation by activating PI3K–AKT–HIF1α signaling in lung adenocarcinoma[49]. As the hub elements of the ceRNAs network, miRNAs exhibited key roles among different RNA transcripts. The miR-671-5p had a protective role in gastric cancer by targeting upregulator of cell proliferation (URGCP)[50]. Meanwhile, miR-671-5p inhibits EMT by directly downregulating FOXM1 in breast cancer[51]. Interestingly, the level of miR-671-5p was not only increased in colon cancer tissue but also increased cell proliferation, migration and invasion by targeting TRIM67[52]，which is contrary to our findings. The same miRNA can regulate multiple mRNA molecules and produce different physiological effects. miR-328-3p was identified in bladder cancer and suppressed cell proliferation, migration and invasion through targeting ITGA5 by inhibiting EMT and inactivated PI3K/AKT pathway[53]. Similar tumor suppression effect was observed in colon cancer[54].

The present study identifies a novel ceRNAs network, which imply that TIMP1 is a potential biomarker underlying the development of CRC, providing new insights for survival prediction and therapeutic target. The progress and limitations of the study should also be recognized. Integrated analysis of multiple to investigate CRC-related circRNAs has hardly been done in previous studies. Nevertheless, what we found in the study may be the tip of an iceberg. The biomarker and pathological mechanism need to be further explored and the result of present CRC related ceRNAs regulatory network are required to verified by clinical trials and molecular experiments.

Declarations

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Authors' contributions

DJK, YFQ and GML conceived of the design of the study. JPH, YMZ, HDW performed and collected the data and contributed to the design of the study. HW prepared and revised the manuscript. All authors read and approved the final content of the manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

This study was approved by the ethics committee of the Tianjin Medical University General Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

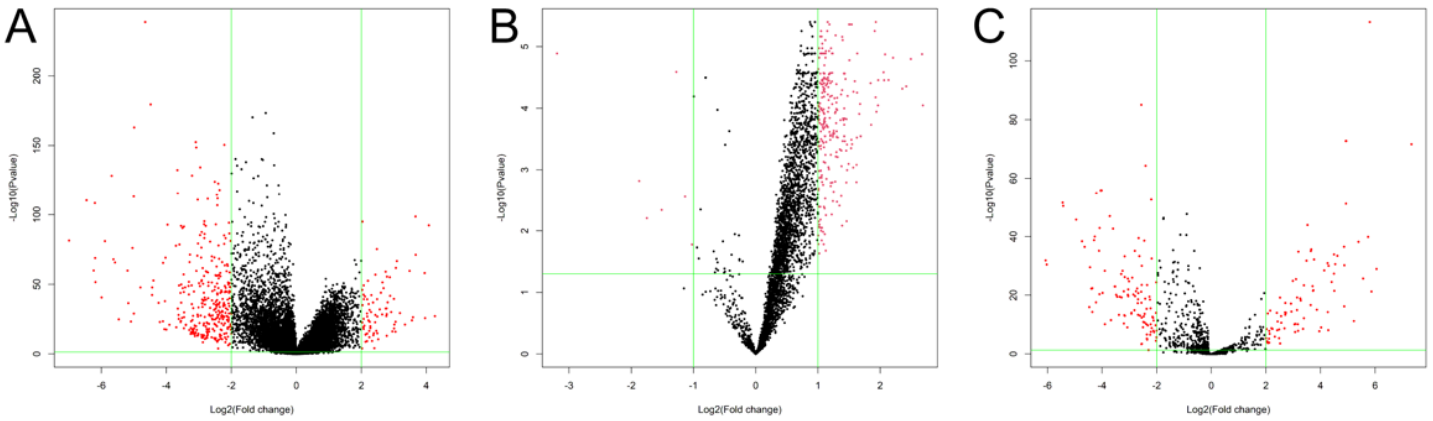

Figure 1

The volcano maps of DERNAs in CRC samples and normal tissue samples. (A) DEGs (B) DEcircRNAs (C) DemiRNAs.

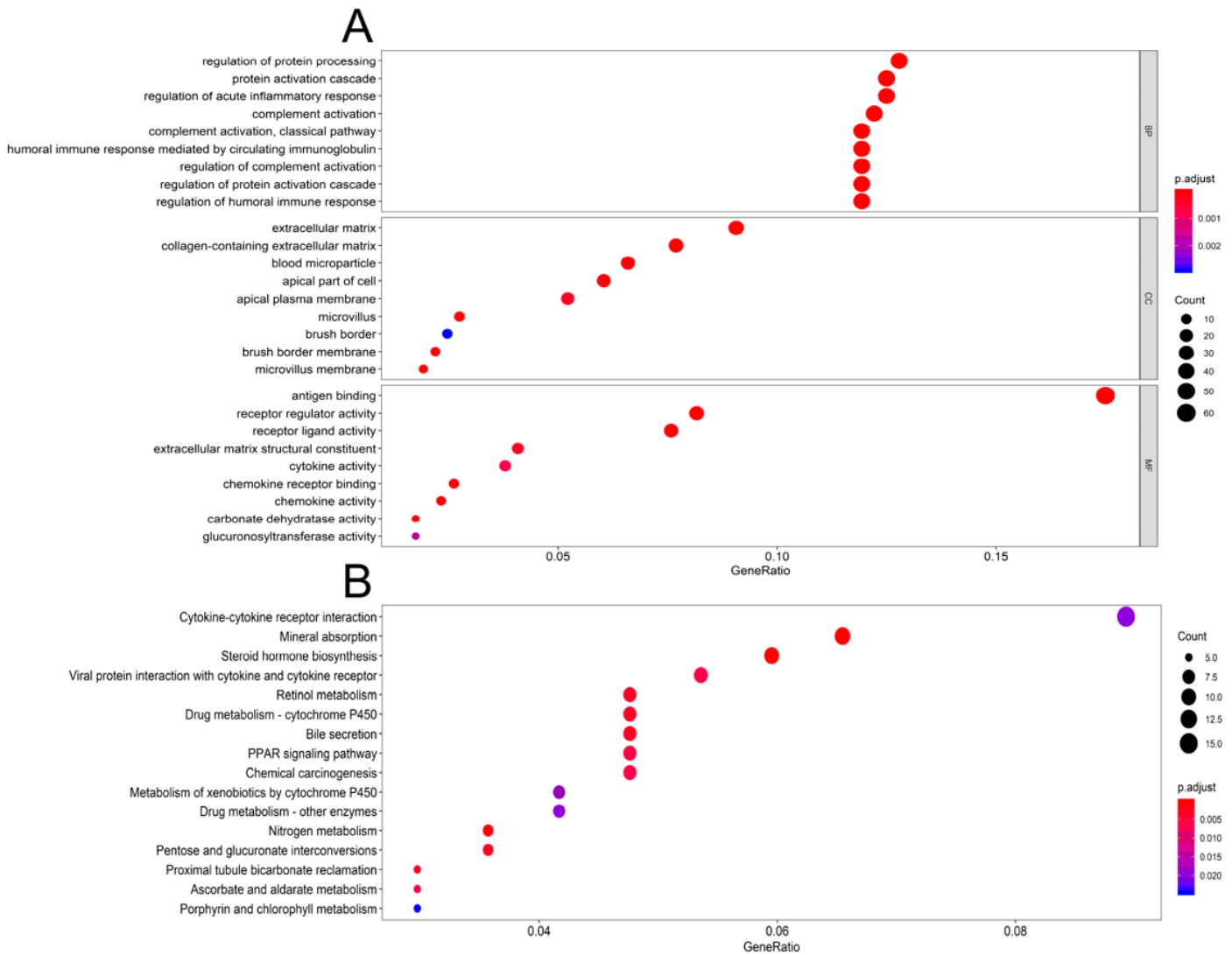

Figure 2

Functional enrichment analysis of DEGs. (A) GO enrichment analyses (B) KEGG-pathway enrichment analyses.

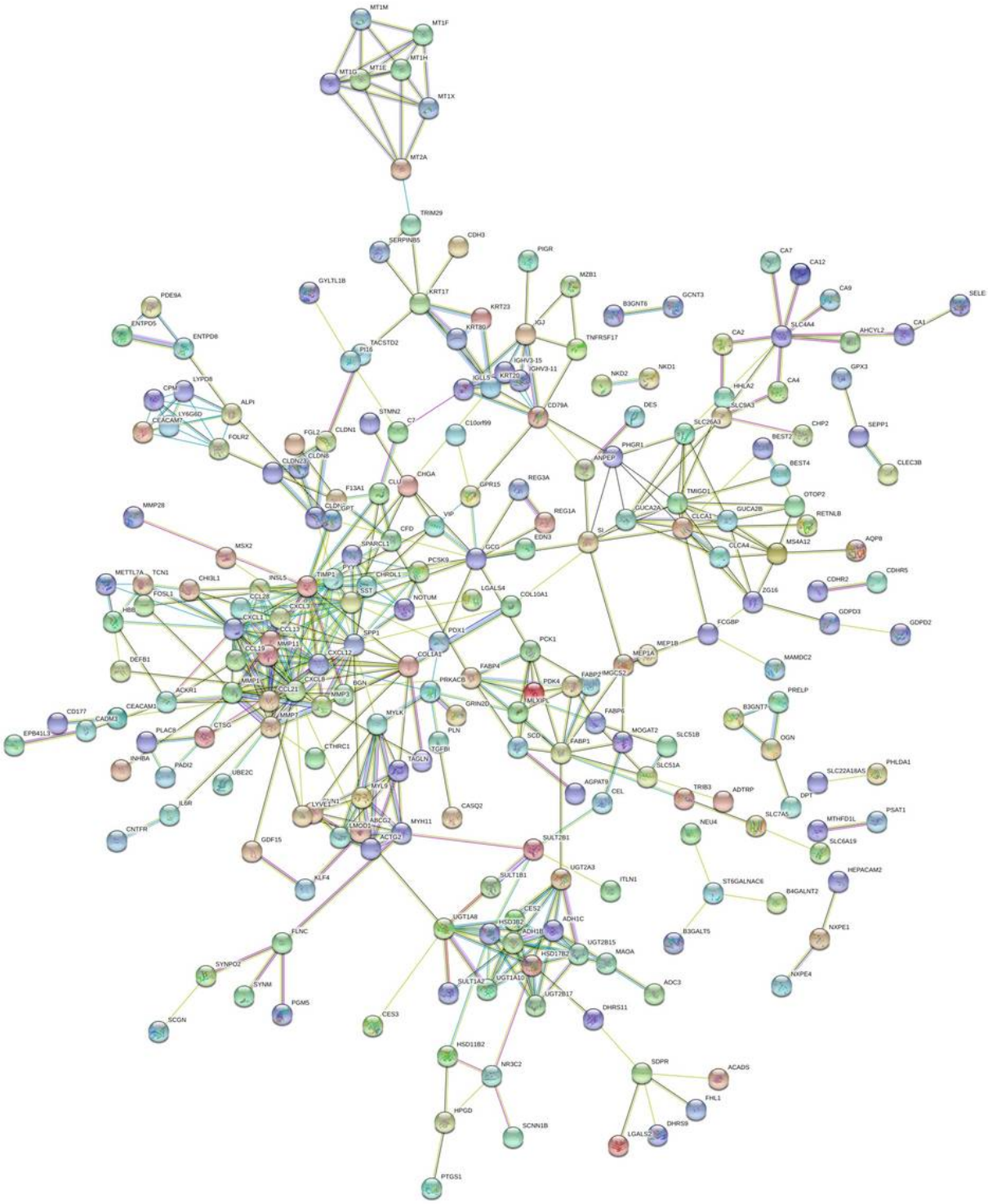

Figure 3

The plot of PPI network of DEGs.

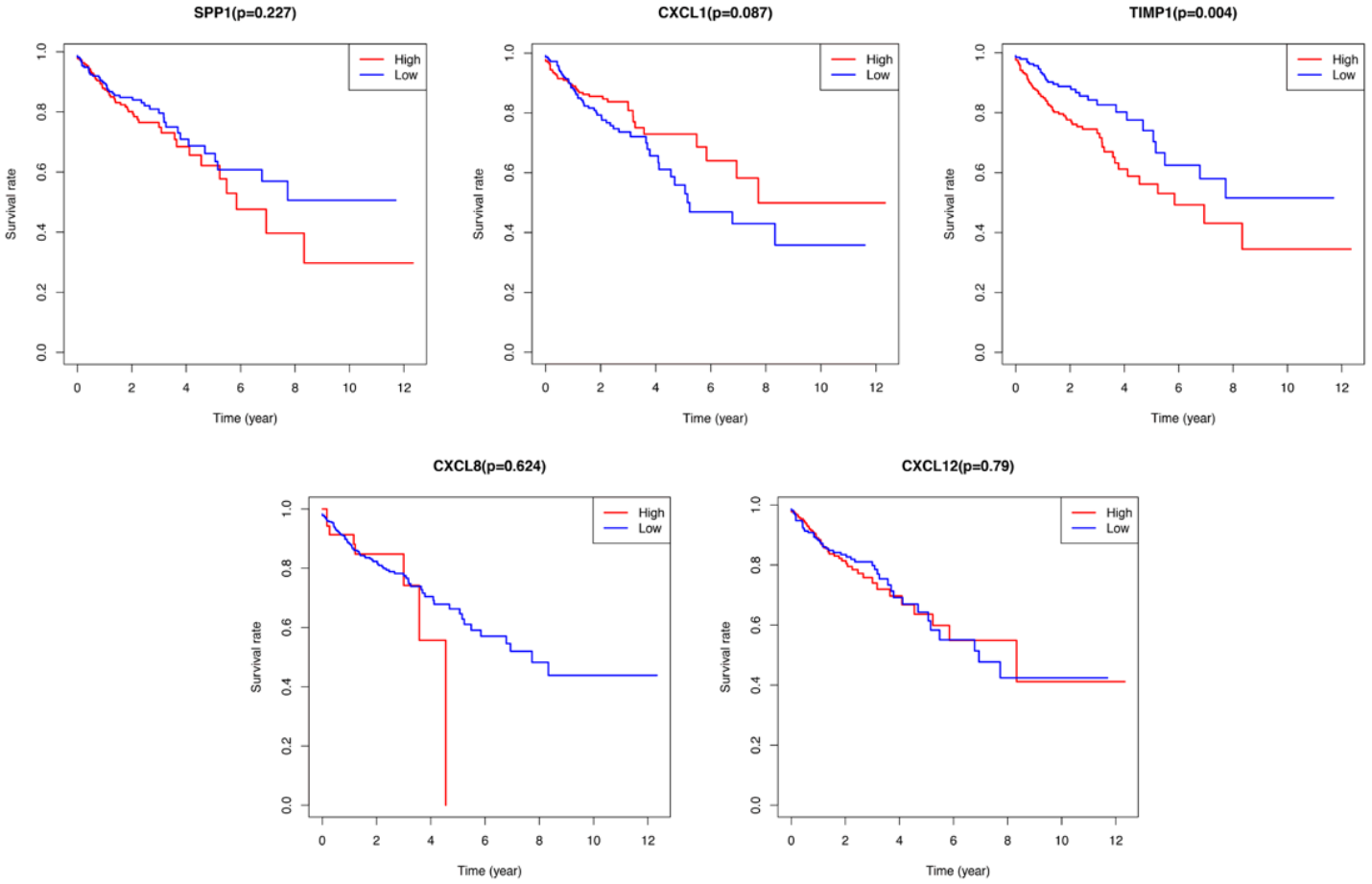

Figure 4

Kaplan-Meier survival plots of the top 5 hub gene.

Figure 5

The ceRNA network of DEcircRNA-miRNA-mRNA in CRC.

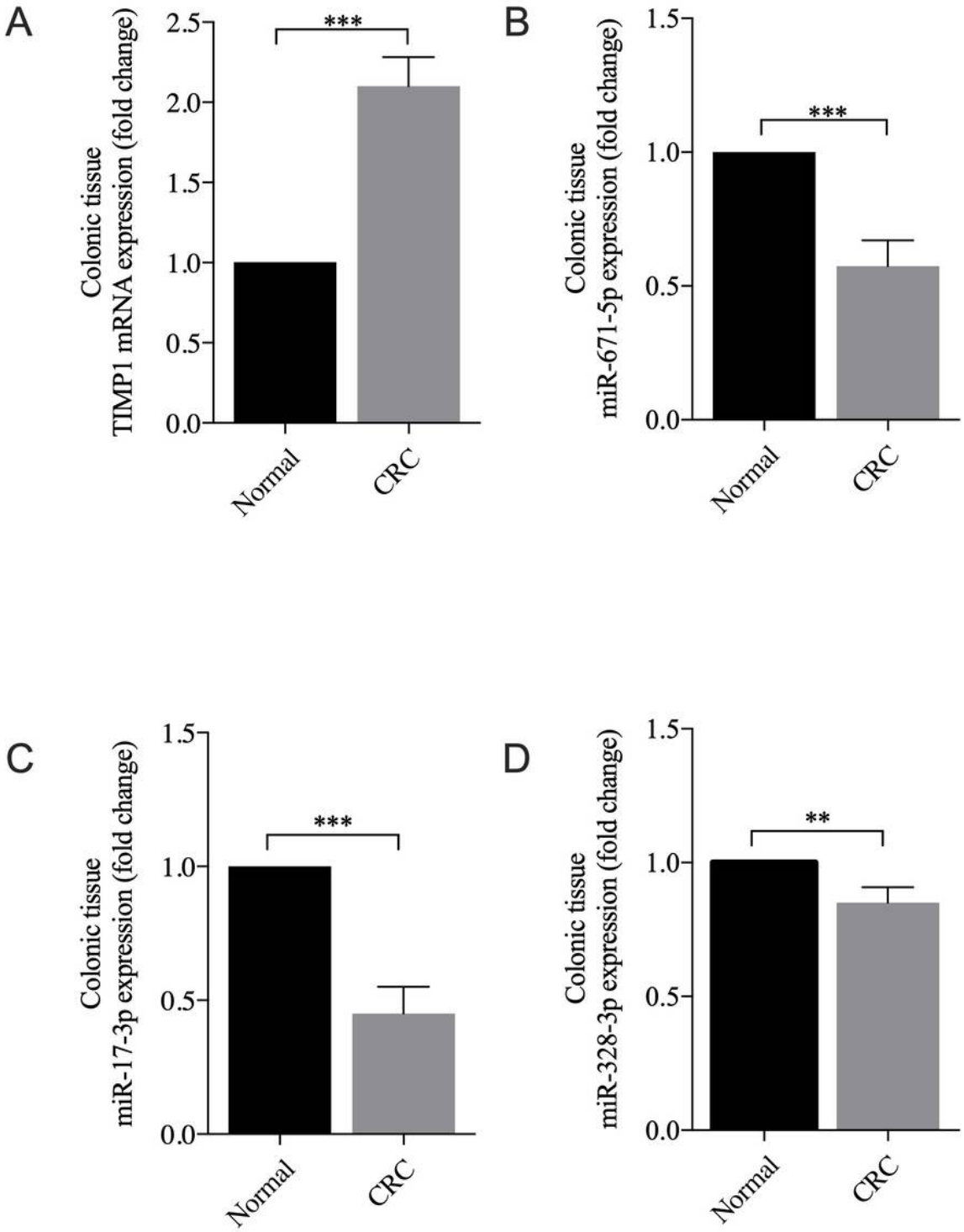

Figure 6

The expression levels of DEmiRNAs and DEGs in human CRC. (A) TIMP1 (B) miR-671-5p (C) miR-17-3p (D) miR-328-3p.