CircRNA-miRNA-mRNA Regulatory Network in Colorectal Cancer

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Primary research

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Abstract

Background: Abnormal expression of Circular RNAs (circRNAs) occurs in the occurrence and progression of colorectal cancer (CRC) and plays an important role in the pathogenesis of tumors. We combined bioinformatics and laboratory-validated methods to search for key circRNAs and possible potential mechanisms.

Methods: Colorectal cancer tissues and normal paracancerous tissues were detected by microarray analysis and qRT-PCR validation, and differentially expressed circRNAs were screened and identified. The circRNA-miRNA-mRNA regulatory network (cirReNET) was constructed, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were used to ascertain the functions of circRNAs in CRCs. In addition, a protein-protein interaction (PPI) network of hub genes which acquired by string and plugin app CytoHubba in cytoscape was established. Validation of expression of hub genes was identified by GEPIA database.

Results: 564 differentially expressed circRNAs which include 207 up-regulated and 357 down-regulated circRNAs were detected. The top 3 up-regulated circRNAs (hsa_circRNA_100833, hsa_circRNA_103828, hsa_circRNA_103831) and the top 3 down-regulated circRNAs (hsa_circRNA_103752, hsa_circRNA_071106, hsa_circRNA_102293) in chip analysis were chosen to be verified in 33 pairs of CRCs by qRT-PCR. The cirReNET include of 6 circRNAs, 19 miRNAs and 210 mRNA. And the targeted mRNAs were associated with cellular metabolic process, cell cycle and glandular epithelial cell differentiation and so on. 12 and 10 target hub genes were shown separately in upregulated circRNA-downregulated miRNA-upregulated mRNA (UcDiUm-RNA) group and downregulated circRNA-upregulated miRNA-downregulated mRNA (DcUiDm-RNA) group. Finally, we may have predicted and discovered several critical circRNA-miRNA-mRNA regulatory axes (cirReAXEs) which may play important roles in colorectal cancer.

Conclusion: We constructed a cirReNET including 6 candidate circRNAs, which were crucial in CRCs, may become potential diagnostic markers and predictive indicators of CRCs, and we may provide a research direction for the pathogenesis of colorectal cancer.

Introduction

Colorectal cancer (CRC) is the fourth most deadly cancer worldwide, with almost 900000 deaths annually [1].With nearly 2.1 million new cases each year, the incidence of CRC is projected to increase to 160 % by 2030.The cost of treating CRC places a huge financial burden on the healthcare system and society as a whole[2].A variety of factors are involved in the development of colorectal cancer. The carcinogenic process of CRC includes environmental factors and genetic factors, among which the environmental factors include high-fat diet, irregular work and rest and mental stress. Genetic factors include the complex accumulation of mutations and epigenetic regulation in key genes[3].With the increasing of high mortality and recurrence rate, it is particularly important to prevent the occurrence of colorectal cancer and explore its pathogenesis. As there is no effective treatment for such a serious disease at the present stage, we can take prevention as the priority and combine prevention with treatment. The morbidity and mortality of CRC can also be reduced through appropriate screening and surveillance [4], it is necessary to explore more reliable markers to find out patients and to implement personalized treatment.

More than 70% of the human genome can be transcribed, and less than 2% of genes encode proteins, with non-coding RNA(ncRNA) accounting for the vast majority of the transcripts[5].Circular RNAs(circRNAs) are ncRNA that have been studied extensively in recent years. Compared with linear RNAs, circRNAs do not have 5’-cap and 3’-poly (A) tail. They form a closed loop structure by covalent bond connecting the head and tail, which makes it difficult to be degraded by exonuclease, and therefore is more stable than linear RNA. CircRNA biogenesis takes place in the nucleus begin with a backsplicing reaction. Then multiple functions are displayed after circRNAs released into the cytoplasm, and the

functions included circRNA could bind to miRNA and RBPs, participate in translation regulation, Protein scaffold, Histone modification, RNA maturation. Moreover, vescicles contains circRNAs were released from cytoplasm may be considered as a biomarker[6]. Currently, a variety of studies have demonstrated that circRNAs can regulate gene expression in a variety of biological processes, participate in the occurrence and development of various diseases, and is involved in pathogenesis of various cancers [7].In the field of reproduction, studies have shown that the expression of circRNA in sperm is second only to that in brain. Analysis of the differentially expressed circRNAs indicates that they could be used as biomarkers for sperm quality evaluation[8].In view of the disease, some researchers found that circFKBP8 and circMBNL1 in whole-blood had clinical diagnostic value for major depressive disorder (MDD), and circFKBP8 could be used for the antidepressive treatment[9].In cancer research, Wang et al.[10] found that circBACH2 was up-regulated in triple-negative breast cancer. Xu et al. [11] thought that Circular RNA hsa_circ_0003288 involved in regulation of hepatocellular carcinoma. It has been shown that abnormal expression of circRNA can accumulate the wrong gene products, the processes will lead to the irreversible occurrence of malignant tumor formation. The mechanism might be circRNAs function as sponges for microRNAs (miRNA) or bind to proteins [12].Therefore, circRNAs may become biological indicators for cancer prediction, diagnosis and treatment.

In the present research, the circRNAs differential expression profile was analyzed using 3 pairs of circRNAs chip in CRCs and six potential key circRNAs were identified by qRT-PCR. The circRNA competing endogenous RNA (ceRNA) network related to CRC was established. GO and KEGG pathway analysis were conducted. In addition, 6 differentially expressed circRNAs were assessed by the receiver operating characteristic (ROC) analysis. Finally, target genes according to circRNA-miRNA-mRNA regulatory network (cirReNET) were analyzed by string database and a plugin app CytoHubba in cytoscape, and the hub genes were screened to show expression of the differences between colorectal cancer and normal paracancerous tissues. Several core circRNA-miRNA-mRNA regulatory axes (cirReAXEs) were found in CRC. Having taken cirReNET into account, our finding may offer a novel insight into the molecular mechanisms of CRCs, and these circRNAs may become key molecules in the diagnosis, prediction and treatment of CRC.

Materials And Methods

Tissue acquisition

33 inpatients with colorectal cancer were enrolled who did not accept any form of chemotherapy, radiation and targeted therapy. They received surgical treatment in the Colorectal Surgery Department of the Affiliated Hospital of Ningxia Medical University during April 2019 to December 2020, respectively. The procedure was approved by the ethics committee and all patients signed informed consent. CRC tumor and normal paracancerous tissues (tissues within 10-15cm adjacent to the carcinoma) were collected and stored in -80℃. The clinicopathological features belong to 33 inpatients (Table 1) are shown. For circRNA chip detection, three pairs of CRC tumor and normal paracancerous tissues were used, clinical characteristic were as following: tumor differentiation was moderate, no lymph node metastasis, and TNM stage (IIB).

Table 1. Clincopathologic characteristics of CRC patients

Variables	Case,n(%)
Age(M±SD，years)	60.39±10.29
Gender	
Female	14
Male	19
Tumor location	
Colon	12
Rectum	21
Lymphatic invasion	
No	19
Yes	14
Venous invasion	
No	23
Yes	10
Nerve invasion	
No	17
Yes	16
Tumor size(cm)	
≤5	30
＞5	3
Malignant degree	
Low/Moderate risk	26
High risk	7

CircRNA Chip Detection

Having homogenized with TRIzol reagent (Invitrogen, United States) by the Mini-Bead-Beater-16 (Biospec, United States), Total RNA of 3 pairs of collected samples were then extracted used RNeasy mini-kit (Qiagen, Germany), while simultaneous digestion was performed with DNase (Baseline Zero DNase, Epicentral, USA).NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, United States) was used to evaluate RNA quantity, and denaturing agarose gel electrophoresis was applied to assessed RNA integrity, as we described before[3].After the required RNA samples were prepared, microarray hybridization was performed according to the standard protocol of ArrayStar. For enriching circular RNAs, Rnase R (Epicentre, Inc.) was used in total RNAs to remove linear RNAs. Then a random priming method (Arraystar Super RNA Labeling Kit; Arraystar) was utilized to amplify and transcribe the enriched circular RNAs into fluorescent cRNA. The fluorescent cRNAs were hybridized by the Arraystar Human circRNA Array V2 (8x15K, Arraystar). The Agilent Scanner G2505C was used to scan arrays after the slides having been washed.

Acquired array images was analyzed by Agilent Feature Extraction software (version 11.0.1.1)[13] . R software limma package was used for quantile normalization and subsequent data processing.

Quantitative real-time PCR

SuperScript III Reverse Transcriptase (Invitrogen) was used to reverse RNA into complementary DNA (cDNA). The forward (F) and reverse (R) primer sequences (Supplementary material Table S1) for qRT-PCR were designed by a software named CircPrimer (Zhong et al., 2018), synthesized by AnHui General biosystems Co., Ltd. (Chinese).The procedure used for qRT-PCR was 95.0℃ for 2 min, and 40 circles of 95.0℃ for 5 s，60℃ for 30s and 95.0℃ for 15 s, 60.0℃ for 1 min and 95.0℃ for 15 s using SYBR Green PCR Master Mix system. RNA levels were normalized to GAPDH expression and 2-ΔΔCT method was used for calculating the fold changes to determine the mRNA expression levels.

CircRNA-miRNA-mRNA Interaction Prediction

These CRCs circRNA microarray results were integrated to screen commonly dysregulated circRNAs. The top 3 up-regulated circRNAs and the top 3 down-regulated circRNAs were chosen for analysis. We used the Cancer-Specific CircRNA (CSCD) to show the fundamental structure of circRNAs. TargetScan and miRanda databases were used to predict the interations of circRNA and miRNA. For miRNA target genes, there datebases which included TargetScan, miRanda v5, and miBase were used, the candidate mRNAs exist in at least two databases simultaneously. Cytoscape software (version 3.6.1) was used to build the cirReNET for visualization.

Gene Ontology (GO) Annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

GO analysis was used to construct and demonstrate meaningful annotation of gene products in multifarious organisms. Biological process (BP), cellular components (CC) and molecular function (MF) were included. KEGG covered molecular interaction and reaction networks in genes. DE mRNAs were mapped to GO analysis and KEGG pathways in order to acquire their function information. Then the candidate mRNA in cirReNET was analyzed by GO and KEGG pathways. GO term (BP, CC and MF) and KEGG pathway were considered have statistical significance ($P < 0.05$, $FDR < 0.05$) in the results.

Protein-Protein Interaction (PPI) Network Construction

The miRDB, miRWalk and TargetScan database were used to predict the mRNAs. For each miRNA, only these target genes predicted by three databases were retained. Target genes of the top 3 up-regulated circRNAs and the top 3 down-regulated circRNAs were analyzed separately by Search Tool for the Retrieval of Interacting Genes/Proteins (string) database to establish the PPI network. The cytoscape plugin app CytoHubba was used to rate target genes, and those with high scores were identified and listed as hub genes. Then the top 30 hub genes were entered into string to draw PPI network.

Validation and Analysis of Hub Genes

The GEPIA 2.0 [14] was used to analysis the hub genes in The Cancer Genome Atlas TCGA (COAD), the GEPIA database is a web server provided profiling and interactive analyses for cancer and normal gene expression. The top 50 hub genes were entered into GEPIA to obtain analysis results. The boxplot was formed with |Log2FC| =1, p-value<0.05 and visualized with log2 (TPM + 1) for log-scale.

Statistical Analysis

The differentially expressed circRNAs in colorectal cancer and normal paracancerous tissues were analyzed by paired t-test. The clinicopathological characteristics of patients with CRCs were assessed using median circRNA expression, and analyzed by Chisquare test to investigate. All data were analyzed by SPSS19.0 statistics software. And P-value (0.05) was used as the cut-off value of statistical significance.

Results

Overview of differentially expressed circRNA in the tissues of patients with CRC

As microarray assay is an efficient way for studying the biological function of RNA, the expression of circRNAs between cancer (CA) and normal control (NC) groups in the CRC were measured. The block diagram represents the normalized intensity of the two groups (Fig. 1A).The variation was assessed using hierarchical clustering analysis and volcano plots. According to the principle of | fold change| > 2, P < 0.05, 564 circRNAs were detected to be differentially expressed in the CA groups and NC groups (Fig. 1B and C). Among the differentially expressed circRNA, 207 and 357 circRNAs were up-regulated and down-regulated, respectively. According to the nature of their source coding genes, the circRNAs were classified into five categories: Among the up-regulated circRNAs,antisense(n = 4),exonic(n = 172),intergenic(n = 4),intronic(n = 13),sense overlapping(n = 14). And the down-regulated circRNAs, antisense(n = 4),exonic(n = 303)，intergenic(n = 4)，intronic(n = 21)，sense overlapping(n = 25)(Fig. 1D) .

Validation and verification by qRT-PCR assay

According to the defined threshold, 16 up-regulated circRNAs (fold change > 3 and P < 0.05) and 16 down-regulated circRNAs (fold change > 5 and P < 0.05) are shown in the cluster heatmap (Fig. 2A) and these circRNAs (Table 2) were manifested. The accuracy of circRNAs microarray data was evaluated by qRT-PCR. The top 3 most up-regulated circRNAs (hsa_circRNA_100833, hsa_circRNA_103828, hsa_circRNA_103831) and the top 3 most down-regulated circRNAs (hsa_circRNA_103752, hsa_circRNA_071106, hsa_circRNA_102293) were chosen for validation and verification via qRT-PCR assay. RT-PCR results showed that the expression level of circRNAs in cancer tissue was consistent with the circRNAs chip data (Fig. 2B and C), manifesting the reliability and correctness of circRNAs chip data.

Table 2
The top 16 significantly up-regulated and top 16 significantly down-regulatedcircRNAs.

circRNA	Style	P-value	FC (abs)	chrom	strand	circRNA_type	GeneSymbol
hsa_circRNA_100833	Up	0.041404417	11.9693427	chr11	+	exonic	FADS2
hsa_circRNA_001586	Up	0.020644494	5.6125946	chr6	-	sense overlapping	HIST1H3D
hsa_circRNA_103831	Up	0.007338473	4.7409963	chr5	-	exonic	HMGCS1
hsa_circRNA_103828	Up	0.023048044	4.1522142	chr5	-	exonic	HMGCS1
hsa_circRNA_104296	Up	0.02754745	4.1136695	chr7	-	exonic	RNF216
hsa_circRNA_400633	Up	0.00796824	3.7916743	chr10	+	exonic	SCD
hsa_circRNA_027446	Up	0.01251238	3.7891116	chr12	+	exonic	HMGA2
hsa_circRNA_407024	Up	0.031582655	3.2868653	chr7	-	exonic	ZNF767P
hsa_circRNA_401518	Up	0.037198732	3.2686815	chr16	-	exonic	TRAP1
hsa_circRNA_062557	Up	0.040687681	3.256663	chr22	-	exonic	CHCHD10
hsa_circRNA_102395	Up	0.041155184	3.2070677	chr19	+	exonic	PTBP1
hsa_circRNA_104893	Up	0.011917176	3.1960212	chr9	+	exonic	PAPPA
hsa_circRNA_065638	Up	0.025681377	3.1094732	chr3	-	exonic	GPX1
hsa_circRNA_003201	Up	0.026106899	3.1040179	chr4	+	exonic	TBC1D14
hsa_circRNA_000676	Up	0.006494205	3.0807421	chr22	+	sense overlapping	L3MBTL2
hsa_circRNA_103140	Up	0.042213331	3.0223647	chr21	+	exonic	PDXK
hsa_circRNA_103752	Down	0.019673168	17.4354314	chr4	-	exonic	LRBA
hsa_circRNA_071106	Down	0.012172022	8.7288597	chr4	+	exonic	ARHGAP10
hsa_circRNA_102293	Down	0.046702335	8.3722472	chr18	+	exonic	MTCL1
hsa_circRNA_005232	Down	0.042405228	8.2508771	chr2	-	exonic	SLC8A1
hsa_circRNA_405040	Down	0.003433548	6.8606691	chr12	+	exonic	PLXNC1
hsa_circRNA_103820	Down	0.004792873	6.5462592	chr5	-	exonic	LIFR
hsa_circRNA_046843	Down	0.001527746	5.9829024	chr18	+	exonic	ANKRD12
hsa_circRNA_042103	Down	0.04696831	5.6131975	chr17	+	exonic	MYOCD
hsa_circRNA_102854	Down	0.025715693	5.5996816	chr2	+	exonic	PDK1
hsa_circRNA_091419	Down	0.029347044	5.5994999	chrX	-	exonic	RPL39
hsa_circRNA_102855	Down	0.013142528	5.5183225	chr2	+	exonic	PDK1
hsa_circRNA_104738	Down	0.035917372	5.3141209	chr9	-	exonic	BNC2
hsa_circRNA_402901	Down	0.035727197	5.264933	chr3	-	exonic	EIF4E3

circRNA	Style	P-value	FC (abs)	chrom	strand	circRNA_type	GeneSymbol
hsa_circRNA_104547	Down	0.036827804	5.2491912	chr7	-	exonic	ESYT2
hsa_circRNA_006473	Down	0.028144483	5.2429886	chr4	+	exonic	ARHGAP10
hsa_circRNA_103729	Down	0.044074879	5.2027097	chr4	-	exonic	PDE5A

The construction of CircRNA-miRNA-mRNA Regulatory Network (cirReNET)

In order to understand the basic structure of the six candidate circRNAs, we used CSCD to predict the fundamental structural modes of the candidate circRNAs (Fig. 3), we can clearly grasp the MRE (microRNA response element), RBP (RNA binding protein) and ORF (open reading frame) structural regions. To further understand the binding function of circRNAs with miRNAs, TargetScan and miRanda databases were used to building circRNA-miRNA interaction network, and the top five targeted miRNAs of there up-regulated and there down-regulated candidate circRNAs (Fig. 4) were shown. According to mechanism circRNAs function as sponges for miRNA, highest context score percentile miRNA were selected to display the potential and detailed sites of circRNA-miRNA interaction (Fig. 5).Then, the cirReNET contain 6 circRNAs, 19 miRNAs, and 210 mRNAs, was visualized via Cytoscape software (version 3.6.1) (Fig. 6). This ceRNA network provides a comprehensive view of the interactions with circRNAs, miRNAs and mRNAs in the pathogenesis of CRC.

GO and KEGG Pathway Analysis

Functional enrichment analysis was used to explore corresponding biological functions.The top 10 enrichment scores for there up-regulated candidate circRNAs are listed (Figs. 7A–C).The GO analysis indicated that the term about biological process terms (BP) with the highest enrichment score was about cellular metabolic process (GO: 0031323).The term about Cellular component terms (CC) was cytoplasm (GO: 0005737) and Nitric-oxide synthase binding (GO: 0050998) for molecular function terms (MF). In addition, the top 10 enriched KEGG pathways are listed (Fig. 7D).The pyrimidine metabolism signalling pathway has the highest enrichment score in the KEGG pathway.

In contrast, the top 10 enrichment scores for there downregulated candidate circRNAs are listed (Figs. 7E–G).The term were related with cell cycle (GO: 0007049) and glandular epithelial cell differentiation (GO: 0002067) for BP. The term about CC with the highest enrichment score was Banded collagen fibril (GO: 0098643) and the term with the highest enrichment score was Protein binding (GO: 0005515) for MF. Respectively, the DNA replication has the highest enrichment score in the KEGG pathway, and the top 10 enriched KEGG pathways are listed (Fig. 7H).

Validation of differentially expressed circRNAs

To verify the expression of six significantly differential circRNAs, 33 pairs of CRCs and normal paracancerous tissues were verified by qRT-PCR. The top 3 up-regulated circRNAs (hsa_ circRNA_100833, hsa_circRNA_103828, and hsa_circRNA_103831) were dramatically up-regulated in CRC tissues (Fig. 8A-C) ($P < 0.05$), and the top 3 down-regulated circRNAs (hsa_circRNA_103752, hsa_circRNA_071106 and hsa_circRNA_102293) were significantly down-regulated in CRC tissues (Fig. 8D-F) $P < 0.05$), compared with normal paracancerous tissues. The results suggested that these circRNAs involved in the pathogenesis of colorectal cancer.

The diagnostic value of circRNAs was evaluated by ROC curve

The ROC curve is often used to measure the ability of a certain index to identify a specific disease, and the value of AUC is an evaluation index to measure the merits of a model.The diagnostic value for CRC of six candidate circRNAs were evaluated,AUC = 0.6860, AUC = 0.8127, AUC = 0.7502, AUC = 0.9945, AUC = 0.9642, AUC = 0.9486 for hsa_circRNA_100833, hsa_circRNA_103828, hsa_circRNA_103831 and hsa_circRNA_103752, hsa_circRNA_071106, hsa_circRNA_102293, respectively (Fig. 9) ($P < 0.05$).The above data indicate that the six circRNAs have high diagnostic efficiency and can be used as indicators for the prediction and diagnosis of CRC disease.

Correlation of circRNA expressions with clinical pathologic features

In order to get the information of the correlation between the 6 candidate circRNAs expression and clinicopathological characteristics, we divided 33 pairs of CRCs tissues into higher circRNA expression group and lower circRNA expression group based on the median circRNAs expression. Then, Chi-square assay was performed to deal with statistical results. The results suggested that hsa_circRNA_100833, hsa_circRNA_103828, hsa_circRNA_103831 and hsa_circRNA_103752, hsa_circRNA_071106, hsa_circRNA_102293 expressions were not relation to gender, tumor size, differentiated degree, lymphatic invasion, venous invasion and Nerve invasion (Fig. 10), the reason might be that the sample size is not large enough.

Construction of PPI network

To understand more about the core interactions among the up-regulated and down-regulated target genes in cirReNET, we used miRDB, miRWalk and TargetScan database to predict target genes for the 5 miRNA for each circRNA according to ceRNA mechanism. Only these genes predicted by three databases were retained. Then functional genes were filtered by string and the PPI networks were visualized. In addition, plugin app CytoHubba in Cytoscape was used to rate target genes according to Maximal Clique Centrality (MCC).Then the top 30 hub genes were entered into string to draw PPI network (Fig. 11), and the top 50 hub genes were listed(Supplementary material Table S2).

Identification of hub genes expression

To understand more information about the expression of predicted target genes in colorectal cancer and normal paracancerous tissues, GEPIA was used to calculate and show the expression of hub genes in TCGA (COAD). We chose the top 50 hub genes to enter into GEPIA to screen(Fig. 12), and the 150 hub genes in the UcDiUm-RNA network, there was higher expression for 12 hub genes(CCL4,APLN,FBXO22,SH3KBP1,CKAP4,PDIA6,RCN1,TP53,CCND1,EZH2,E2F2,CASP3) in the CRC group as compared to normal paracancerous tissues group. And the 150 hub genes in the DcUiDm-RNA network,10 hub genes (RNF217,KLHL5,UNKL,PTGER3,FBXO32,TRIM9,KCTD7,KLHL42,SRSF11,RBM5)were lower expression in the CRC group in the DcUiDm-RNA network. In addition, we may have predicted and discovered several critical cirReAXEs that play important roles in colorectal cancer (Fig. 13), the next step is further experimental confirmation.

Discussion

CRC accounts for a significant proportion of cancer deaths and is one of the most commonly diagnosed malignancies[2].Multiple studies have shown that the occurrence and development of colorectal cancer involved the differential expression of various circRNAs in normal tissues and cancer tissues. CircRNAs are newly discovered and ubiquitous endogenous ncRNAs [15].Multiple studies have shown that circRNAs function as sponges for miRNAs and regulate the corresponding target genes expression to affect dozens of diseases. Han et al. found that the up-regulated circRNA hsa_circ_0071036 in pancreatic cancer can promote the oncogenic process through sponging miR-489, which can be used as an indicator of tumor prediction and prognosis[16].Liu et al. thought that circular RNA CircMTO1

suppressed proliferation and metastasis of osteosarcoma through miR-630/KLF6 axis[17].Abnormal expression of circRNAs can lead to malignant behavior and malignant progression of various tumors[2]. CircRNAs have been thought to regulate miRNAs and play an important role in ceRNA networks. Of course, according to the current studies, abnormally expressed circRNAs participate in the process of CRC carcinogenesis and regulate the expression of related genes and proteins. Li et al. have confirmed that CircRNA_101951 regulated EMT pathway could promote migration and invasion of CRC cells[18]. RNA sequencing of another study [19] revealed circDDX17 played as tumor suppressor in CRC. There also have been many studies about circRNAs in colorectal cancer, such as circ_0115744 sponge miR-144 to regulate the metastasis of CRC[20]. CircNSUN2/miR-296-5p/STAT3 axe was under control of Aloperine to inhibit proliferation and promote apoptosis of CRC cells [21]. Circ102049 was identified as a core member by CircRNA profiling in colorectal liver metastasis [22]. In general, current studies on circRNAs in colorectal cancer mainly focus on the regulatory mechanism of miRNA sponge.

To investigate the differential expression of circRNA in CRC, circRNA chip detection was chosen to profile and demonstrate 3 pairs tissues belong to patients with colorectal cancer in the present study. According to the principle of $|\text{fold change}| > 2$, $P < 0.05$, 564 circRNAs were detected to be differentially expressed in the CA groups and NC groups. Among the differentially expressed circRNA, 207 and 357 circRNAs were up-regulated and down-regulated in CRCs tissues, respectively. Then, three up-regulated circRNAs (hsa_ circRNA_100833, hsa_circRNA_103828, hsa_circRNA_103831) and three down-regulated circRNAs (hsa_circRNA_103752, hsa_circRNA_071106, hsa_circRNA_102293) were identified to confirm consistency with the expression of the chip results. Meanwhile, for a fuller explanation of the function of the cirReNET in CRCs, a network consists of 6 circRNAs, 19 miRNAs, and 210 mRNAs was plotted by software named cytoscape. Differentially expressed circRNAs in the CRC were annotated through a cirReNET, a potential ceRNA mechanism has been explored and demonstrated.

In our network, there were several studies showed many miRNA which were reported before participated in mechanism of tumorigenesis. Such as hsa-miR-665 is involved in proliferation, invasion and epithelial-mesenchymal transition of gastric cancer cells[23].Hsa-let-7b-5p can inhibit the migration and invasion of glioma cells and block the cell cycle[24].hsa-miR-411-5p,hsa-miR-448 mediated the invasion, migration and proliferation of lung cancer and breast cancer cells, respectively[25, 26]. hsa-miR-141-3p[27]、hsa-miR-30a-5p[28]、hsa-miR-145-5p[29] were involved in the regulation of the pathogenesis of various tumor cells. Also, there were many targeted genes involved in the mechanism of tumor genesis, such as AKT1[30],ErbB2[31],ITGB7[32],BAX,MAPK,FBXL12[33],CCL7[34] and so on. These genes also play very important roles in cell proliferation, apoptosis, movement, T-cell differentiation, adhesion, and polarity formation.

The functions of these target genes were then assessed and analyzed with the GO analysis. For target genes corresponding to the 3 up-regulated-circRNAs, GO enrichment analysis manifested many crucial biological processes included in these genes, such as cellular metabolic process, biosynthetic process and so on. Meanwhile KEGG pathway analysis provided a deep insight of the pathogenesis of CRC.KEGG pathway analysis was performed to show some vital pathways, include IL-7 signaling pathway, Glycerophospholipid metabolism, Histidine metabolism et al. For other 3 down-regulated-circRNAs, GO enrichment analysis revealed target genes involved in regulating cell cycle, Glandular epithelial cell differentiation. KEGG pathway analysis showed that DNA replication, mismatch repair and TGF-beta signaling pathway might be the mechanism of the development of CRC.

Afterwards, 33 pairs of CRC tissues and normal paracancerous tissues were collected to verify and validate the expression of identified 3 up-regulated circRNAs and 3 down-regulated circRNAs in our research. The analysis results show that high diagnostic values were involved in all of these circRNAs, especially for 3 down-regulated circRNAs. On the other hand, there were not associated with gender, tumor size, and malignant degrees, lymphatic invasion, venous

invasion, nerve invasion in CRCs. The reasons for the lack of correlation with clinical features may be that the sample capacity was not large enough. The microarray and qRT-PCR data as well as the subsequent bioinformatics prediction indicated that these 3 up-regulated and 3 down-regulated identified circRNAs might be the key circRNAs involved in the mechanisms of carcinogenesis in CRCs, and could be used as potential novel diagnostic predictive and prognostic biomarkers in patients with CRCs.

In order to further understand the core interactions among the up-regulated and down-regulated target genes in cirReNET, the string was used to filter functional genes and the PPI networks were visualized. And differentially expressed up-regulated and down-regulated hub genes were shown by GEPIA, we may have found several core cirReAXEs in CRCs. such as has_circRNA_100833-hsa-miR-124-5p-APLN axe, hsa_circRNA_103831-hsa-let-7c-5p-TP53 axe, hsa_circRNA_071106-hsa-miR-6830-3p-UNKL, These core cirReAXEs may participate in major regulation in the pathogenesis of CRC.

It should be pointed out that our study did have some minor flaws. Firstly, Only 33 patients were enrolled, the sample size is relatively small. In addition, the analysis of the relationship between the clinicopathological features and the expression of circRNAs requires the support of large clinical cases. There is one more point, I should touch on that we only conducted a network based on identified 3 up-regulated circRNAs and 3 down-regulated circRNAs in our work, other circRNAs may be also involved in regulation in mediating the development of colorectal cancer, but we have not explored. The last but not the least, our paper is a simple descriptive analysis of the expression of circRNAs in colorectal cancer, but the function and mechanism of each circRNA in CRCs have not been verified and discussed in detail. In our study, circRNAs were validated with clinical samples, evaluated as biomarkers and analyzed by bioinformatics accordingly. In addition, several critical cirReAXEs which may play important roles in colorectal cancer were proposed. Therefore, the study about underlying mechanisms of circRNAs and the development of related processes in the CRCs are ongoing in our laboratory. In our future work, we also need to further focus on finding out the deeper mechanism of specific circRNAs in CRC through related biological experiments.

Declarations

Conflict of Interest

All authors have reviewed the final version of the manuscript and approve it for publication. The manuscript has not been published in whole or in part nor is it being considered for publication elsewhere. The authors have no conflicts of interest to declare.

Author Contributions

GX and LL conceived, significantly guided in the design, conception, analysis, and interpretation of the findings of this study. DJ, YQ, HW, SW, LL, XX collected CRCs tissues and normal paracancer tissues, extracted RNA, verified RNA quality, and performed reverse transcription with random primer method.circRNAs was verified via qRT-PCR by LL. GX, LL, and DJ analysed the experimental results. LL, YQ and HZ conducted bioinformatics analysis and edited the manuscript. All authors contributed to the article and agreed to the submitted version.

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Ethics approval and consent to participate

These studies involving human participants were reviewed and approved by the Ethical Committee of General Hospital of Ningxia Medical University (approval no. KYLL-2021-37) .

Patient consent for publication

The patients/participants have provided their written informed consent to participate in this study.

Availability of data and materials

All data and materials could be found in our published paper.

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Figures

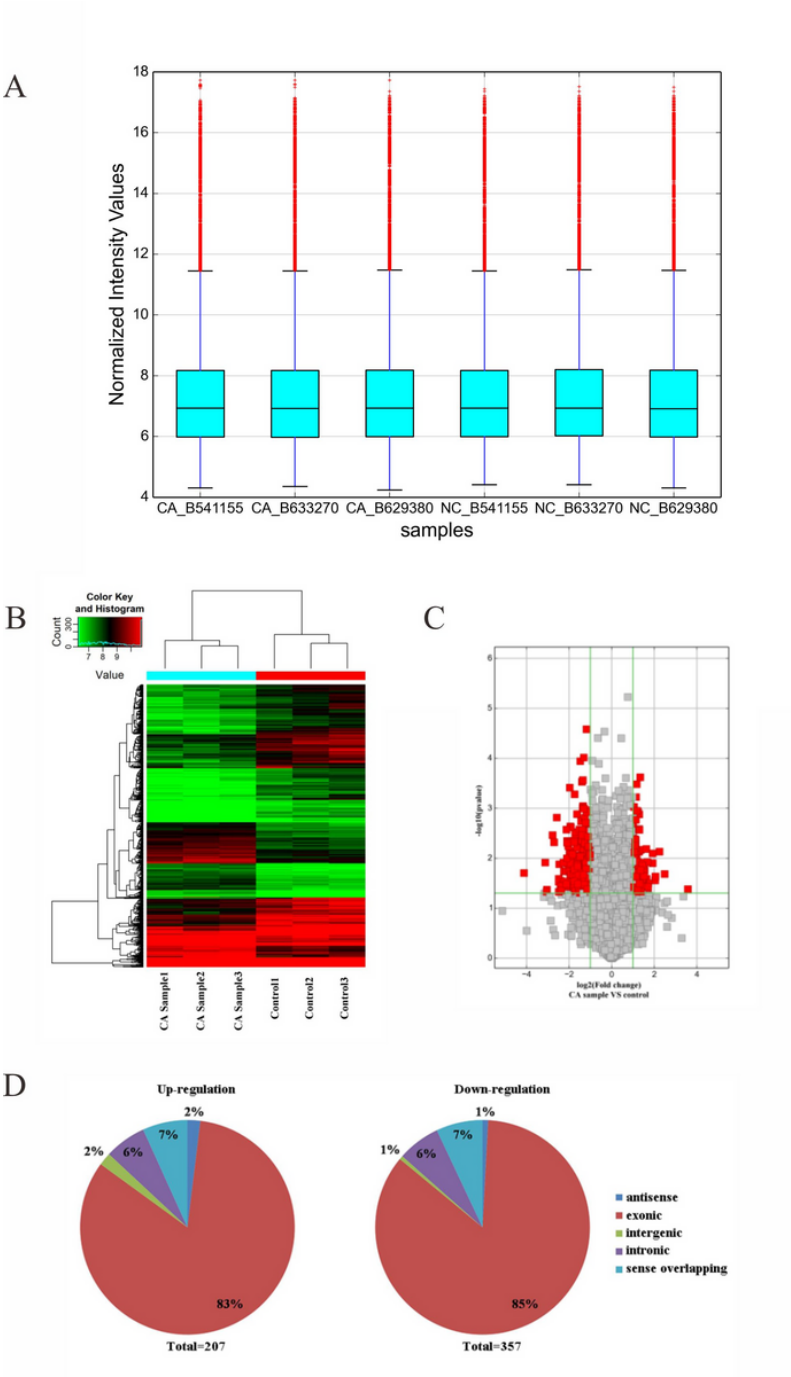

Figure 1

Characterization of circRNA expression in CRC cells and control cells. (A) Boxplot represents the data quality of each chip is reliable after data standardization. (B)Hierarchical clustering shows a distinguishable circRNA expression profile. (C) Volcano plot of the differentially expressed circRNAs. (D) The origin of up-regulated and down-regulated circRNAs. The red squares represent differentially expressed circRNAs with statistical significance.

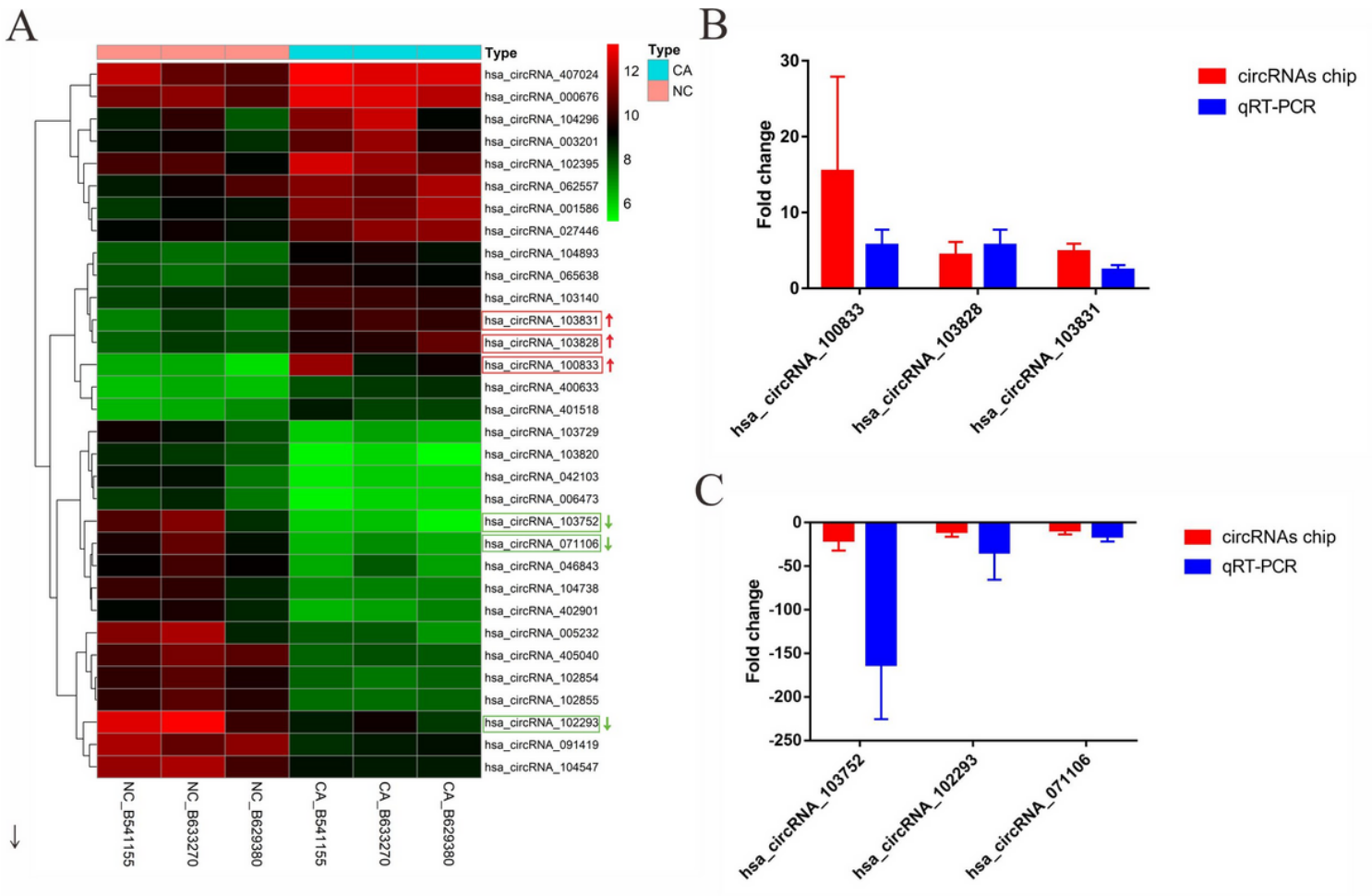

Figure 2

The cluster heatmap of circRNAs chip and histogram of Arraystar human circRNAs chip versus Quantitative Real-Time PCR comparison.(A) Chip analysis of the top sixteen most up-regulated and down-regulated circRNAs. (B) Arraystar human circRNAs chip versus qRT-PCR.GAPDH was used to normalize for measuring circRNA expression levels. NC, normal tissues. CA, cancer tissues.

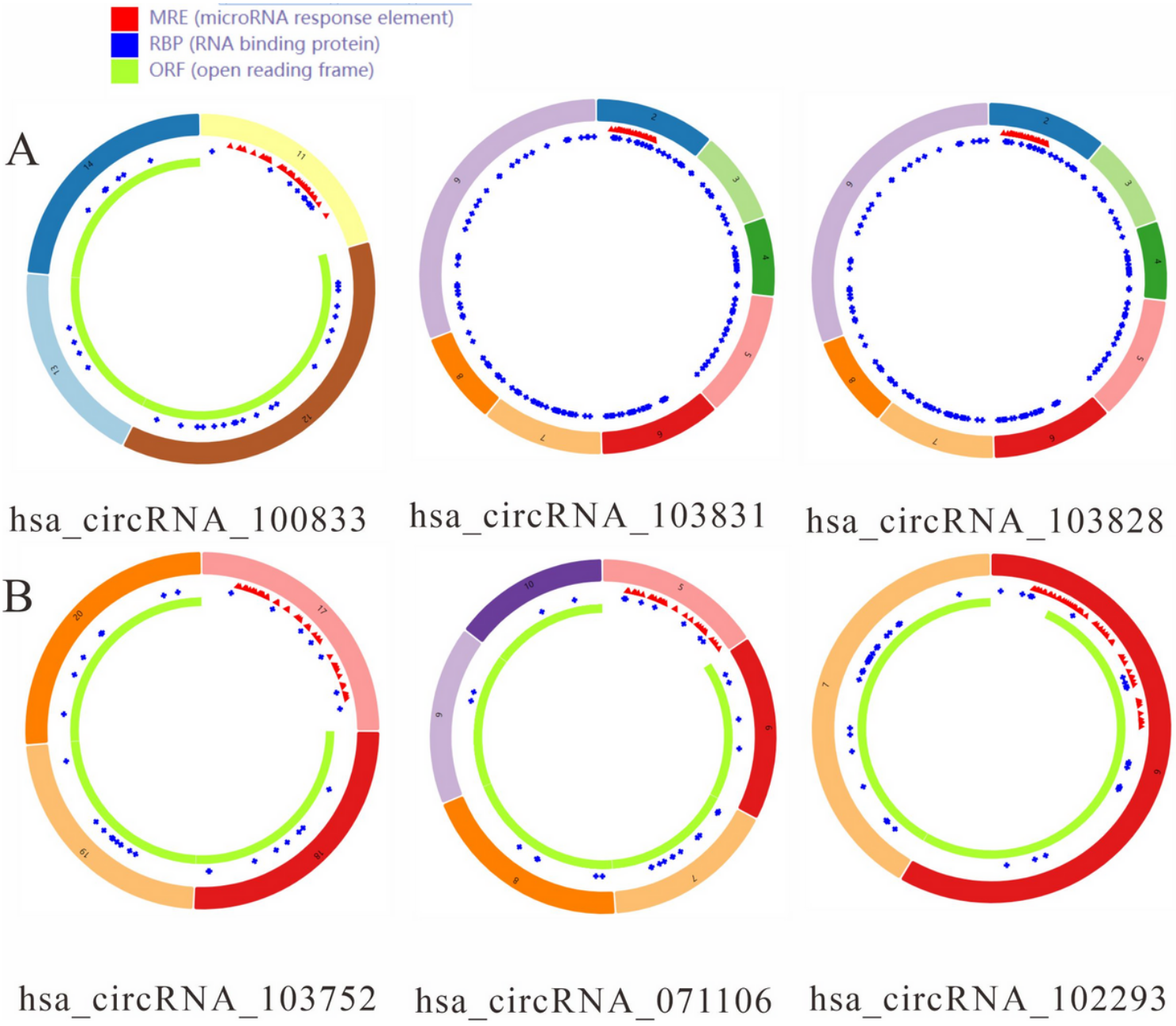

Figure 3

The basic structural patterns of 3 up-regulated circRNAs and 3 down-regulated circRNAs predicted by CSCD. (A) hsa_circRNA_100833, hsa_circRNA_103831, hsa_circRNA_103828. (B) hsa_circRNA_103752, hsa_circRNA_071106, hsa_circRNA_102293.The microRNA (MRE) is represented in red. The RNA binding protein (RBP) is represented in blue. The open reading frame (ORF) is represented in green.

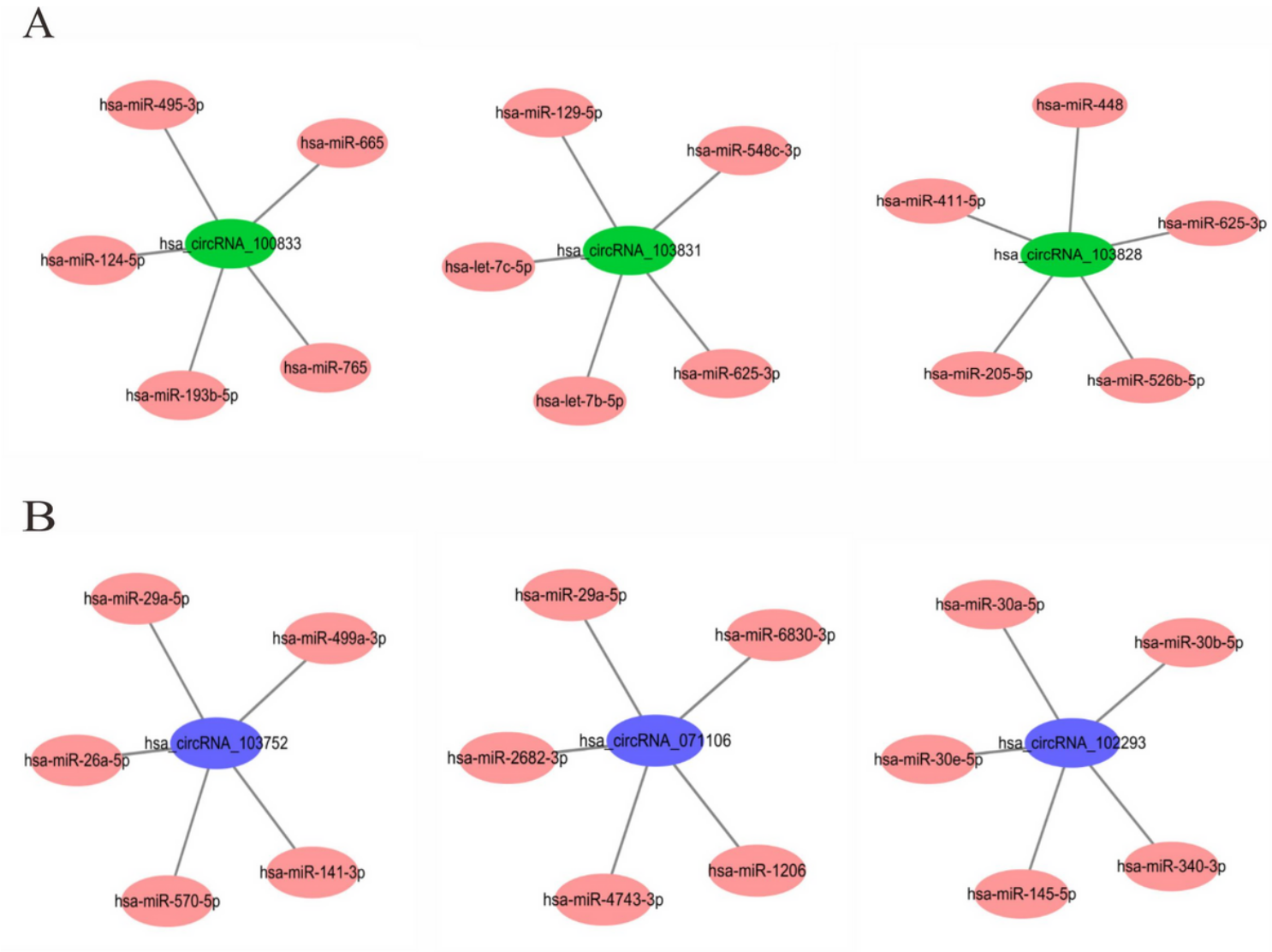

Figure 4

The top five targeted miRNAs for circRNA-miRNA regulatory network.(A) Up-regulated circRNAs. (B) Down-regulated circRNAs.

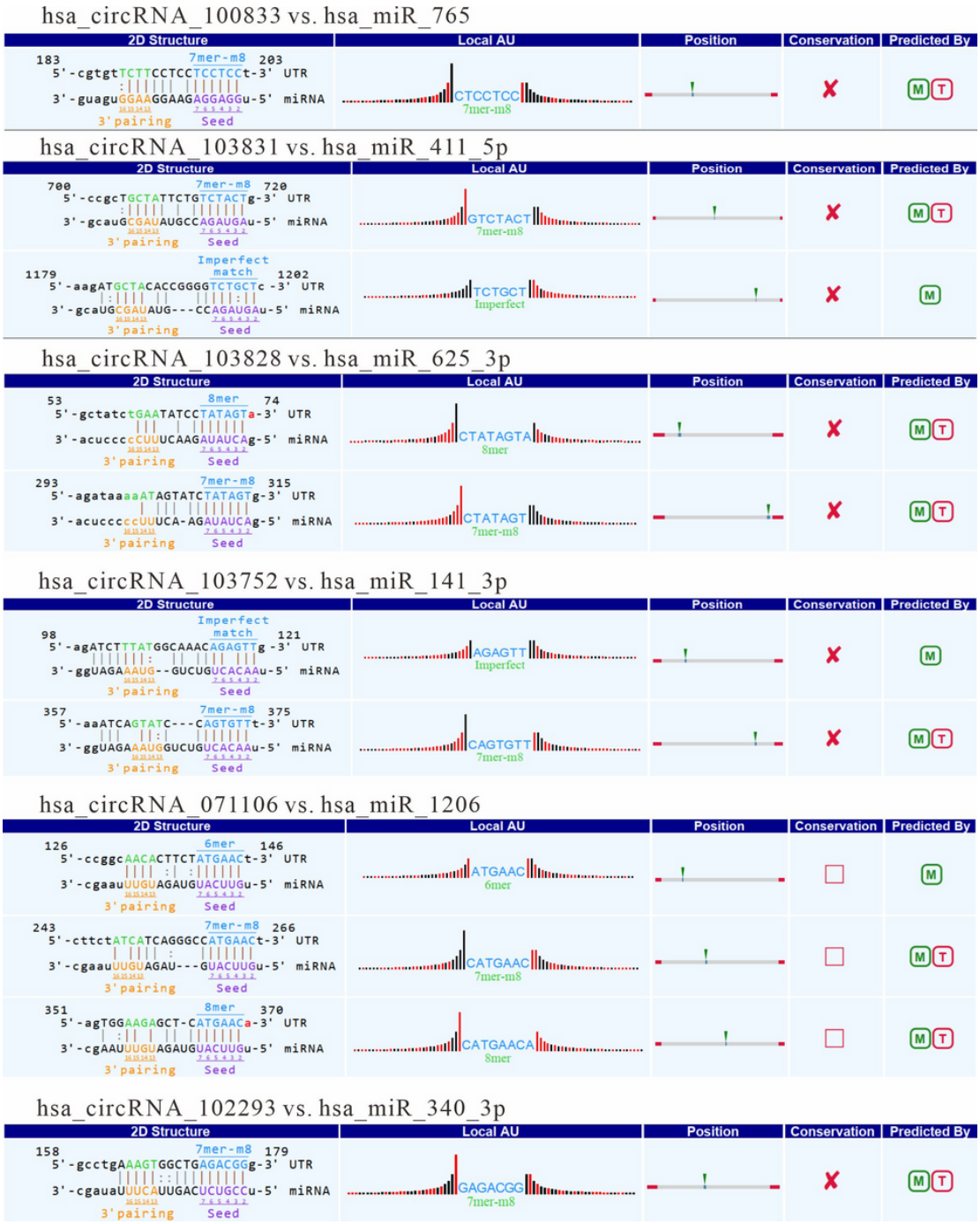

Figure 5

The details of circRNA-miRNA potential interaction sites. MiRNA Binding Sites, circRNA and miRNA Binding secondary structure.

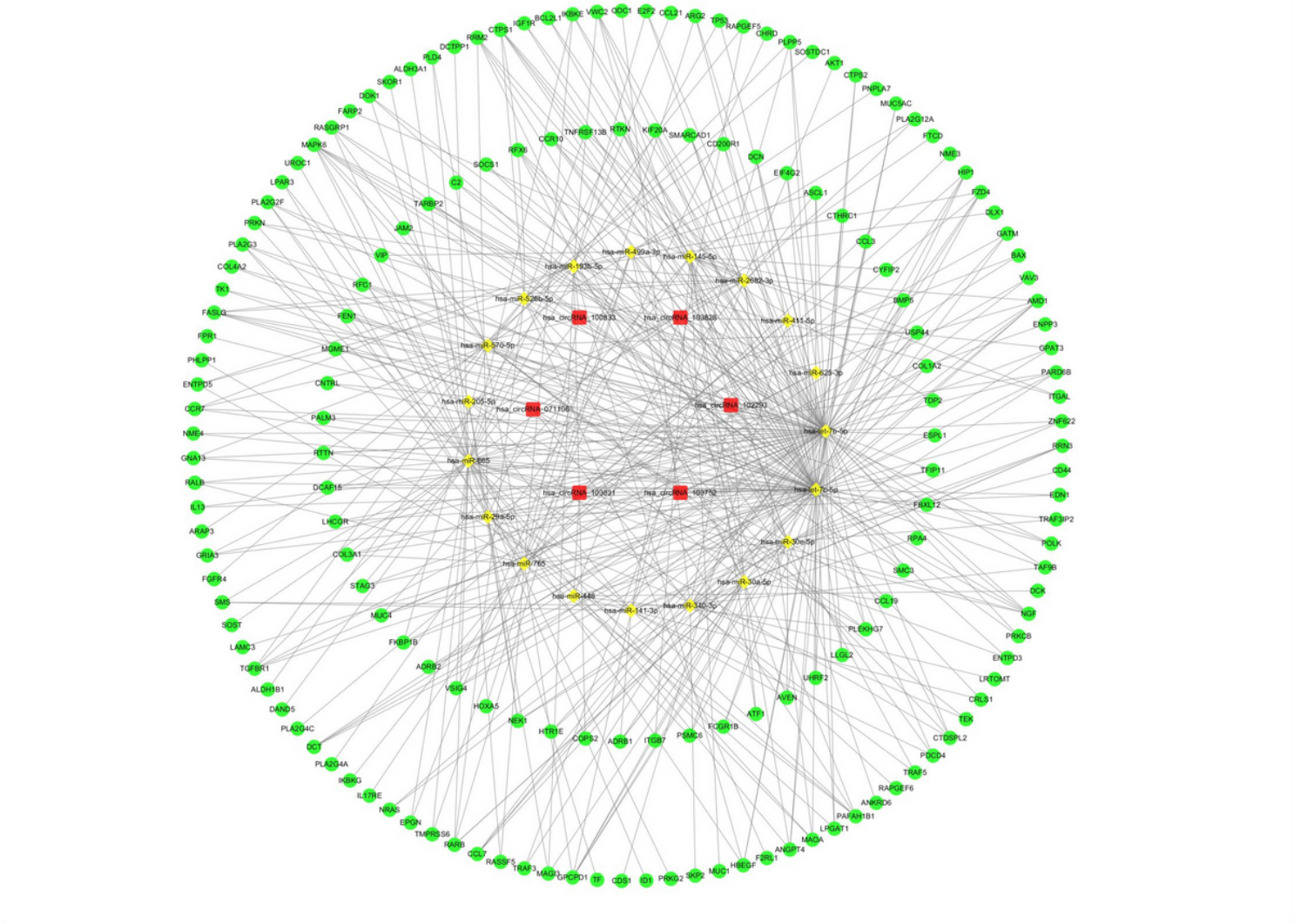

Figure 6

circRNA-miRNA-mRNA regulatory network (cirReNET) in the CRC tissues. The cirReNET included 6 circRNAs, 19 miRNAs and 210 mRNAs. CircRNAs are represented in red and presented as square, miRNAs are represented in yellow and presented as diamond, and mRNA are represented in green and presented as circle.

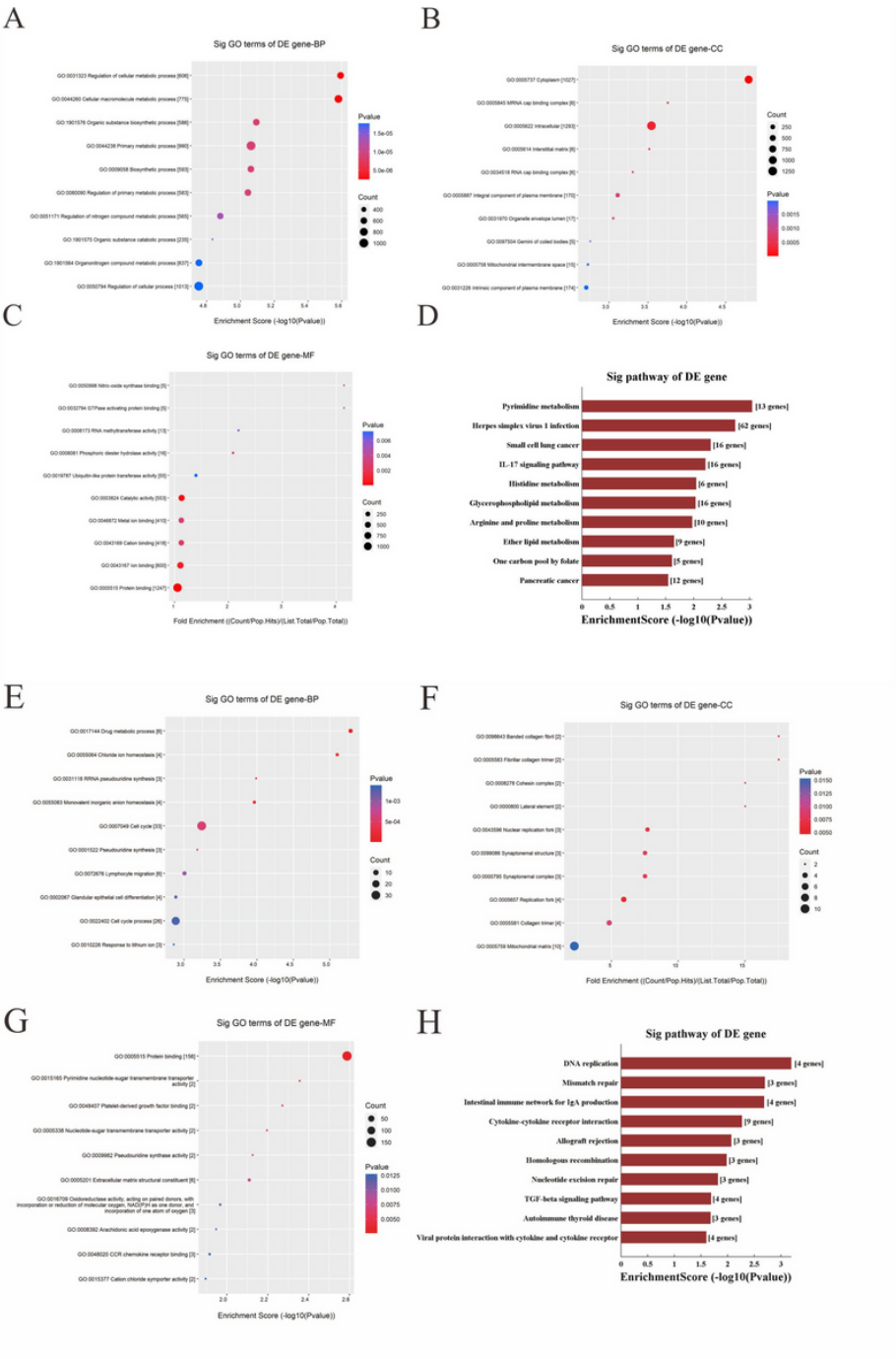

Figure 7

GO and KEGG pathway analysis for 3 up-regulated circRNAs and 3 down-regulated circRNAs. (A–C & E-G) GO annotation of targeted genes with the top 10 enrichment scores for BP, CC, and MF, respectively. (D&H) The top 10 enriched KEGG pathways. Enrichment score was calculated as -log10 (P-value). (A-D)Up-regulated circRNAs. (E-G)Down-regulated circRNAs. GO, gene ontology. KEGG, Kyoto Encyclopedia of Genes and Genomes. BP, biological process. CC, cellular component. MF, molecular function.

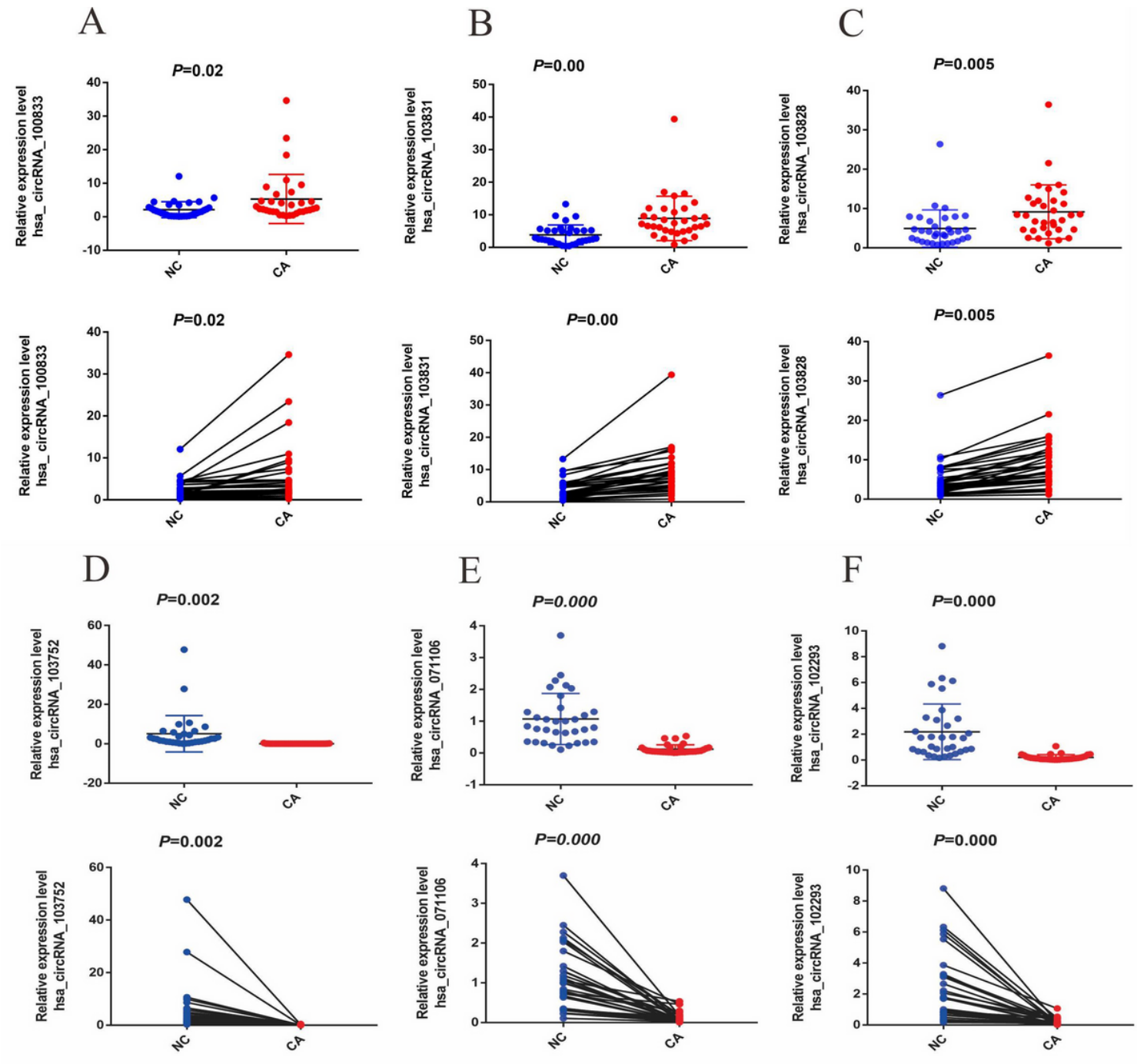

Figure 8

The expression of up-regulated and down-regulated circRNAs of 33 CRC tissues (A) hsa_circRNA_100833 (B) hsa_circRNA_103831 (C) hsa_circRNA_103828 (D) has_circRNA_103752 (E) hsa_circRNA_071106 (F) hsa_circRNA_102293. GAPDH was used to normalize for measuring circRNA expression levels. P<0.05 indicated a difference in comparison between the two groups, and P<0.001 indicated a significant difference.

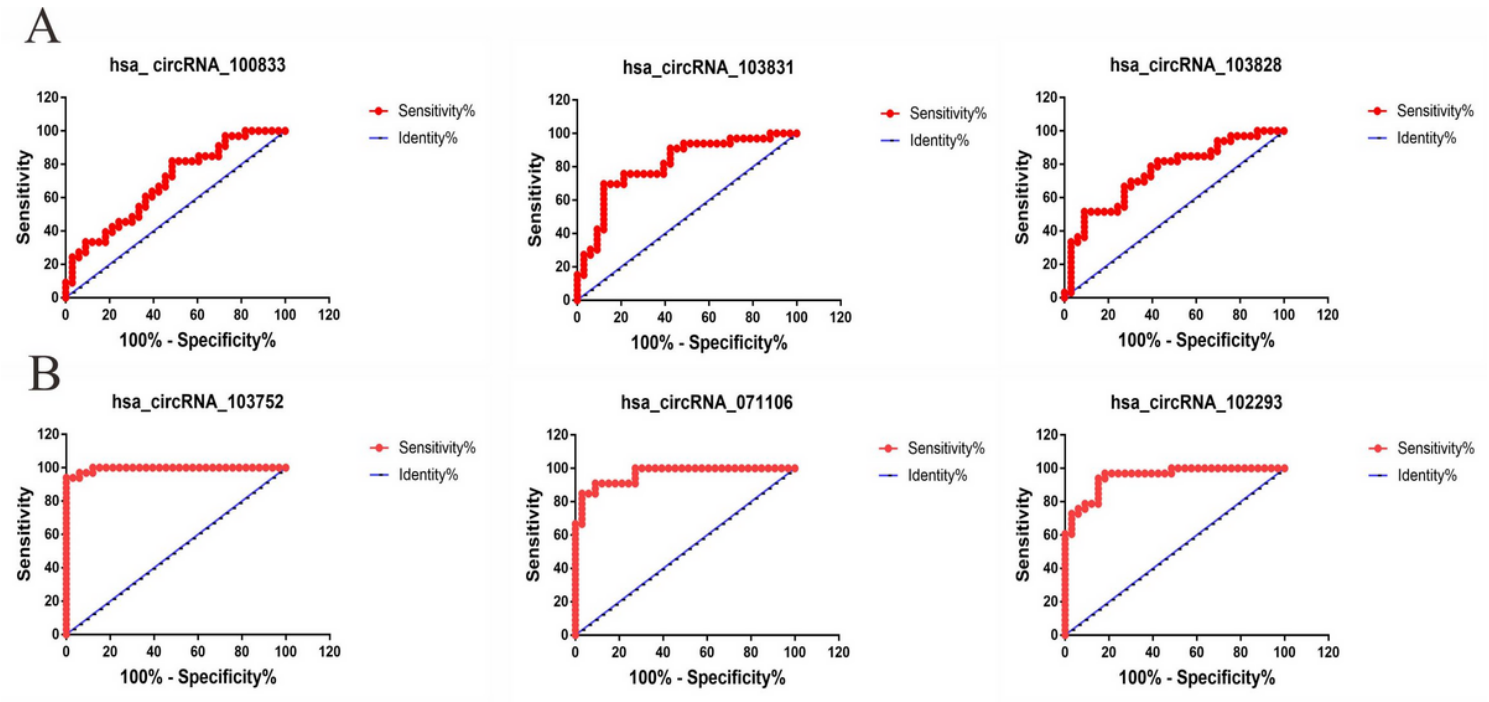

Figure 9

The ROC curves of 3 up-regulated and 3 down-regulated circRNAs in CRC. (A) The diagnostic value of three up-regulated circRNAs presented by ROC curve. (B) The diagnostic value of three down- regulated circRNAs presented by ROC curve.

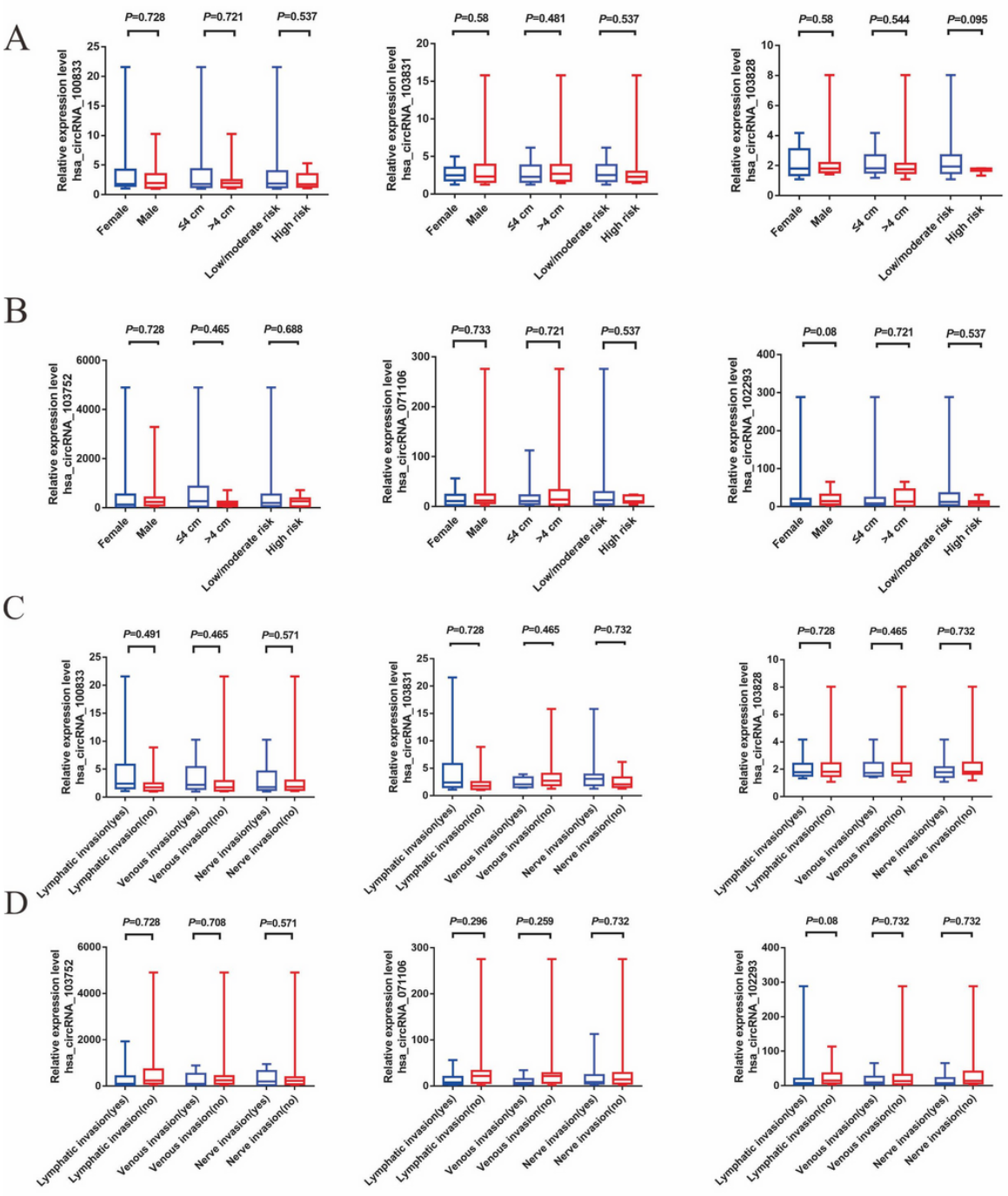

Figure 10

Correlation of six candidate circRNAs with clinicopathological parameters showed by box pots. (A&C) Up-regulated circRNAs. (B&D) Down-regulated circRNAs.

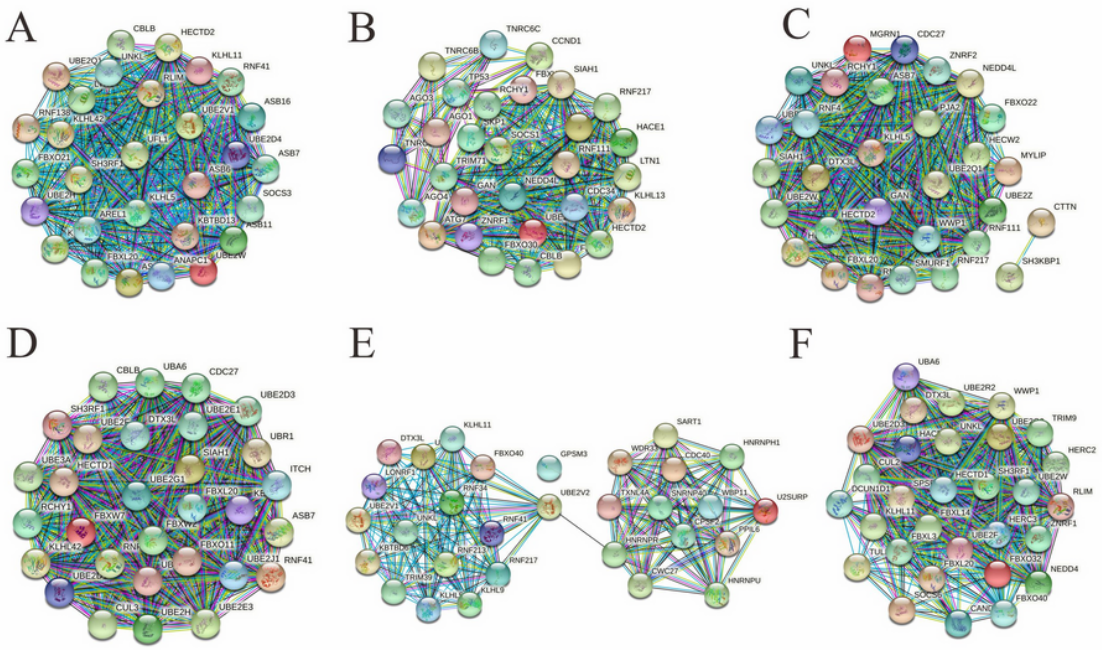

Figure 11

PPI networks consisting of hub genes belong to 3 up-regulated circRNAs and 3 down-regulated circRNAs. (A)The top 30 hub genes belong to hsa_circRNA_100833. (B)The top 30 hub genes belong to hsa_circRNA_103831.(C)The top 30 hub genes belong to hsa_circRNA_103828.(D)The top 30 hub genes belong to hsa_circRNA_103752.(E)The top 30 hub genes belong to hsa_circRNA_071106.(F)The top 30 hub genes belong to hsa_circRNA_102293.

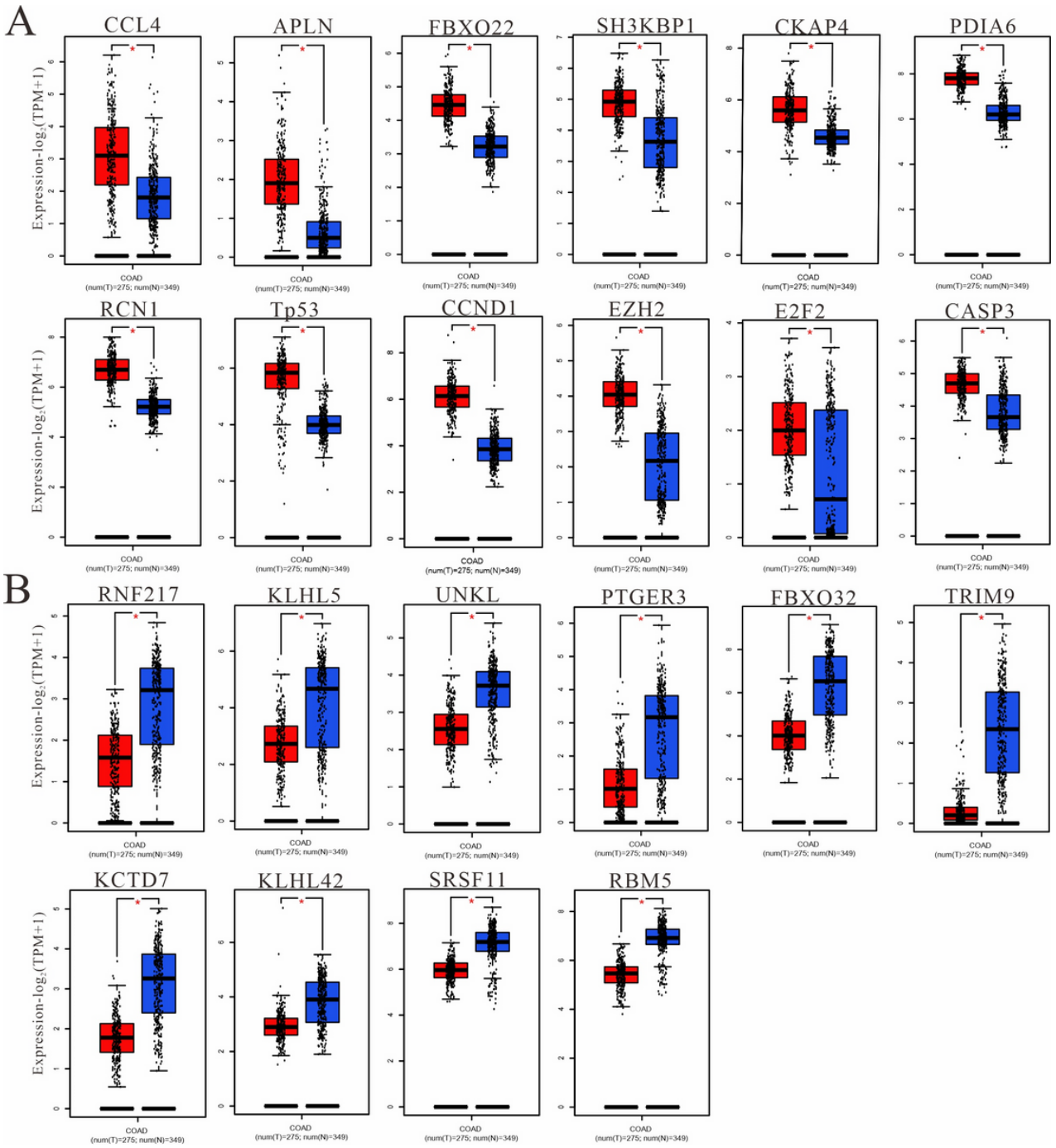

Figure 12

Hub gene expression. (A) Up-regulated hub genes via GEPIA analysis in cancer tissues. (B) Down-regulated hub genes via GEPIA analysis in cancer tissues. "*"represent P<0.05P<0.05 indicated a difference in comparison between the two groups.

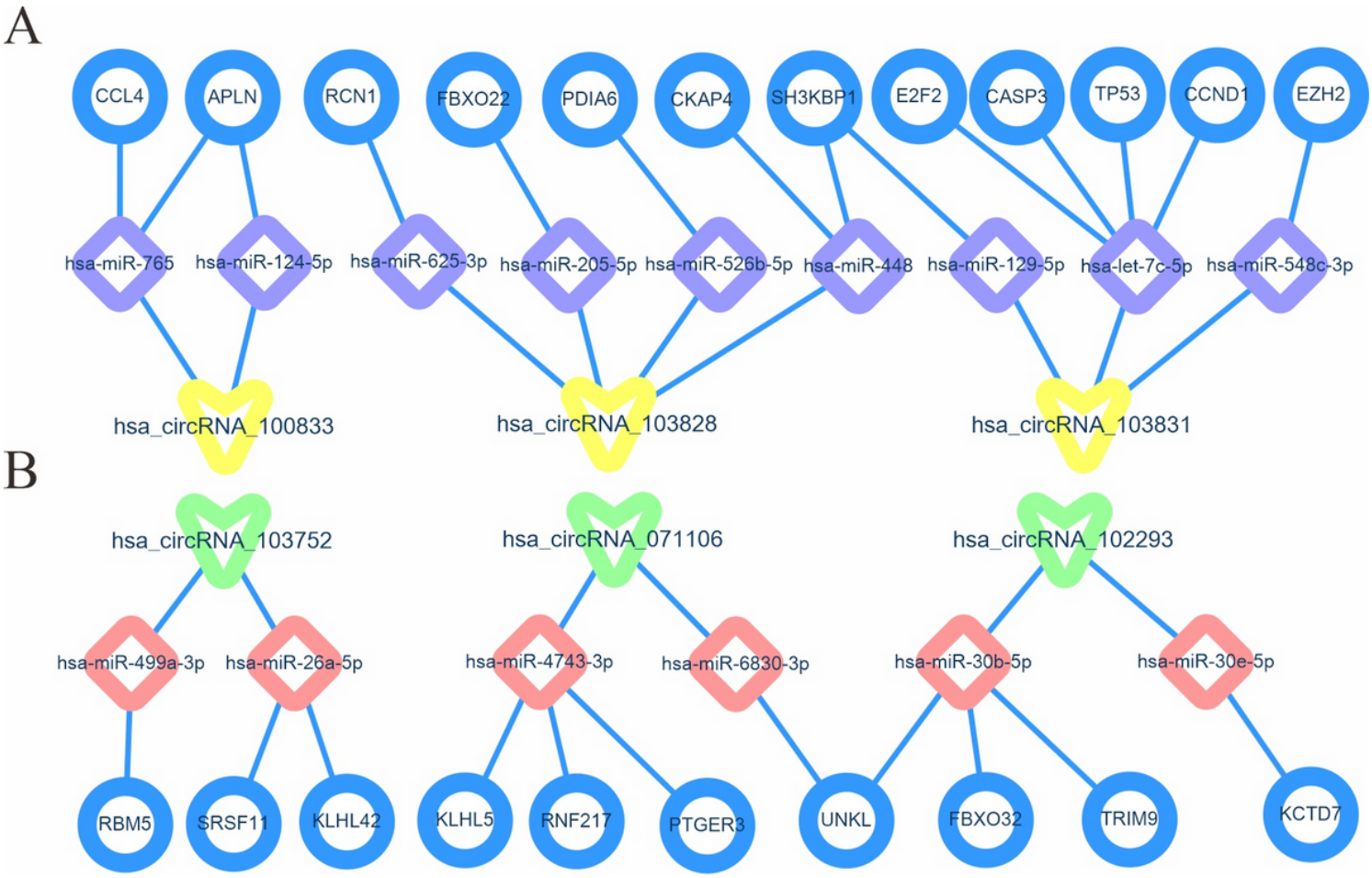

Figure 13

Prediction of core circRNA-miRNA-mRNA axes (cirReAXEs) in the CRC. (A)Core networks for 3 up-regulated circRNAs. (B)Core networks for 3 down-regulated circRNAs.

Supplementary Files

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- SupplementaryMaterial.doc