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ORIGINAL RESEARCH

Evaluation of Diagnostic and Prognostic Value of hsa_circ_0084927 and Analysis of Associated ceRNA Network in Colorectal Cancer

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Object: This study aims to analyze the differentially expressed circRNA in colon adenocarcinoma (COAD) and evaluate its diagnostic and prognostic value. Analyze associated circRNA-miRNA-mRNA network in COAD.

Methods and Materials: Real-time quantitative PCR (RT-PCR) was used to verify differentially expressed circRNA in COAD tissues and cells; Receiver operator characteristic (ROC) and Cox regression analysis were used to evaluating its diagnostic and prognostic value; Meanwhile we conducted CCK-8, invasion, and migration experiments in cell lines to explore the function of circRNA. In addition, a competitive endogenous RNA (ceRNA) network was established using bioinformatics methods to explore its prognostic value and potential functional mechanisms.

Results: Our study found that hsa_circ_0084927 is highly expressed in COAD tissues and cell lines. Plasma hsa_circ_0084927 can be used as a diagnostic marker for COAD patients; hsa_circ_0084927 can promote the proliferation, migration and invasion of COAD cells. In addition, we effectively constructed a ceRNA: network has_circ_0084927/miR-106b-5p/VEGFA. The ceRNA network indicates that hsa_circ_0084927 may affect the prognosis of COAD through the regulation of cell cycle, apoptosis and other pathways.

Conclusion: Our research results indicate that hsa_circ_0084927 has a cancer-promoting effect and may be used as a circulating tumor marker for COAD prognosis. In addition, this study proposes a new ceRNA network to provide new insights for the targeted therapy of COAD.

Keywords: circRNAs, ceRNAs, biomarker, colon adenocarcinoma, prognosis

Introduction

Colon adenocarcinoma is one of the most common tumors worldwide.[1,2] However, many COAD patients have advanced metastases at the time of initial diagnosis, and early diagnosis of tumors can greatly reduce the incidence of tumors. At present, the treatment of colon cancer is mainly surgical resection, supplemented by chemotherapy. Patients with metastatic colon cancer tend to have a poor prognosis, with a high recurrence rate and a 5-year survival rate of less than 40%.[3] Early diagnosis and therapeutic intervention play a crucial role in colon cancer prognosis. In recent years, improved targeted drugs and other precision treatments based on tumor-specific targets have achieved breakthroughs and better curative effects in many tumors. For example, the FDA granted Osimertinib "Breakthrough Therapy" designation for the first-line treatment of patients with metastatic EFGR mutation-positive non-small cell lung cancer. At present, the drugs used for molecular targeted therapy of breast cancer include human epidermal growth factor receptor 2 (HER2)-targeted Lapatinib, mammalian target of rapamycin (mTOR) targeted Everolimus. Currently, the further progress of targeted therapy drugs (cetuximab, panitumumab, etc.) is limited due to drug resistance and individualized selection of colon cancer patients. Thus, there is a lack of more effective and recognized diagnostic

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and prognostic biomarkers and therapeutic targets. Therefore, it is of far-reaching significance to study its mechanism and screen out specific biomarkers and therapeutic targets.

Circular RNA (circRNA) is a new type of endogenous non-coding RNA that is widely present in eukaryotic organisms and can be stably expressed. CircRNA is a closed-loop structure whose particularity makes it not susceptible to degradation.[4–6] CircRNAs can be used as miRNA sponges,[7] protein sponges,[8] transcription regulators,[9] or even peptide coding sequences, indicating that they have the potential to be a target of cancer treatment drugs.[10,11] Therefore, circRNA is considered a potential biomarker, and some of them (such as circHIPK3 and circRNA0003906[12–14]) have been identified as COAD diagnostic and prognostic biomarkers. Recent studies have shown that circRNA can bind to proteins or translate peptides and proteins to perform biological functions,[15,16] and circRNA in the nucleus can act as a transcriptional activator or inhibitor to regulate transcription. But the most common mechanism of circRNA is that it acts as a miRNA sponge, affecting biological processes such as cell proliferation and apoptosis.[17] For example, circRNA-ACAP2 binds to hsa-miR-21-5p to increase transfer protein 1 to promote the proliferation of COAD cells.[18] Therefore, revealing the function of circRNA and elucidating its regulatory mechanism has high scientific and clinical value. Although there has been a lot of research on some circRNAs in COAD, the mechanism exploration of circRNA in COAD is still in its infancy.

This study first analyzed the RNA-seq data of colon cancer cells in our previous sequencing results (GSE189908). Subsequently, we verified the expression of circRNA in colon cancer cell lines and colon cancer tissues, initially explored its biological functions. Then, we constructed a circRNA-miRNA-mRNA regulatory network in colon cancer, and further predicted the possible role of circRNA in colon cancer. Our research helps to determine potential new targets for colon cancer treatment and provides a certain basis and reference for the follow-up study of the role of circRNA in colon cancer.

Materials and Methods

Sequencing Analysis

We selected 6 colon cancer cell lines (3 high-metastatic cell lines and 3 low-metastatic cell lines) for whole-transcriptome high-throughput sequencing to detect RNA expression (GSE189908).

COAD Tissue Specimens

The 96 pairs of tissue specimens used in this study were provided by Guangxi Medical University Cancer Hospital (including colon cancer tissue and adjacent normal tissue). The study was approved by the Ethics Committee of Guangxi Medical University Cancer Hospital, and 96 patients signed the relevant informed consent form (LW2022020) and complies with the Declaration of Helsinki. The inclusion criteria were as follows: COAD patients who were treated at our study center from 2010 to 2015 (all diagnosed by histopathology) did not receive any treatment (chemotherapy, traditional Chinese medicine, and immunotherapy) before surgery, and did not have gastrointestinal diseases and other tumors. Meanwhile, clinical case data and surgical specimens were collected. Postoperative follow-up was carried out by outpatient or telephone. The deadline for follow-up was April 1, 2021.

Cell Culture and Transfection

Human normal colonic epithelial cell lines NCM460 and COAD cells (including HCT116, SW480, DLD1, SW620 and RKO) are purchased at ATCC (American Type Culture Collection). When the cell density reaches 60% to 70%, si-circ is transfected into colon cancer cells using Invitrogen™ Lipofectamine™ RNAiMAXfection Reagent.

Real-Time PCR

Using TRIzol to extract total RNA and reversed it into cDNA following the 20 ul system (PrimeScript RT kit and gDNA rubber Kit, Takara Japan); FastStart Universal SYBR Green Master (ROX) (Roche, Germany) was used for real-time quantitative polymerase chain reaction (qRT-PCR) analysis. The ΔΔCT method (GAPDH standardization) was used to calculate the expression level of circRNA. The primer sequence is as follows: hsa_circ_0084927, Forward 5'-CCTAGCACT AGAGGACCACAA-3', Reverse:5'-GTTCCATCTTGCTGCACCAG-3'. hsa-miR-106b-5p, Forward GCGCGTAAAGT GCTGACAGT; Reverse AGTGCAGGGTCCGAGGATT. VEGFA, Forward GGAGGAGAGAGAAGAGAGAGAGG;

REVERSE: TGGTGGAGAGAGAGGAGAGAGAGG. FORWARD for MECP2: CAGAGGCAGAAAGAAGAC; Reverse AATACATCATACTACCCAGAGC. ITCH's Forward tctcagccccgagtagct; Reverse tggtggtggtggtgaaggtc. CEA level was detected using CEA ELISA Kit (Ze Ye Biotech, China). siRNA was purchased from Guangzhou Ruibo, China, the sequence information is as follows: si-h-hsa_circ_0084927_TAAAATTGCTGGTGCAGCA.

CCK-8 Experiments Detect Cell Proliferation

After transfection 48 h, the transfected cells were inoculated in a 96-well plate with 2000 cells per pore for CCK8 experiments. Add 10 microliter cck8 solution (Zomanbio, China) to the cell and last for 2 hours at 37 degrees C. Detect the absorbance (D) value at 450 nm wavelength with a microplate reader.

Scratch Experiments and Transwell Chamber Experiments Detect Cell Migration

After 48 hours of cell transfection, use a scratch tool to draw a straight line in a 6-well plate, replace the serum-free medium, and place it under an inverted microscope to capture the scratch distance at 0h and 24h, and calculate the relative migration ability of the cells by calculating the migration rate.

Transwell Chamber Experiments Detect Cell Invasion

Place 50L of Matrigel diluent in the upper chamber of the transwell Chamber and place it in the 37°C incubator overnight. After 48 hours of transfection, the cells were seeded in the upper chamber of the transwell chamber at 200 ul per well, and the lower chamber was added to DMEM medium containing 10% FBS. After 24 hours of routine incubation, use a cotton swab to gently wipe the matrigel and cells in the upper chamber, fix with methanol, stained with crystal violet, and take pictures and count.

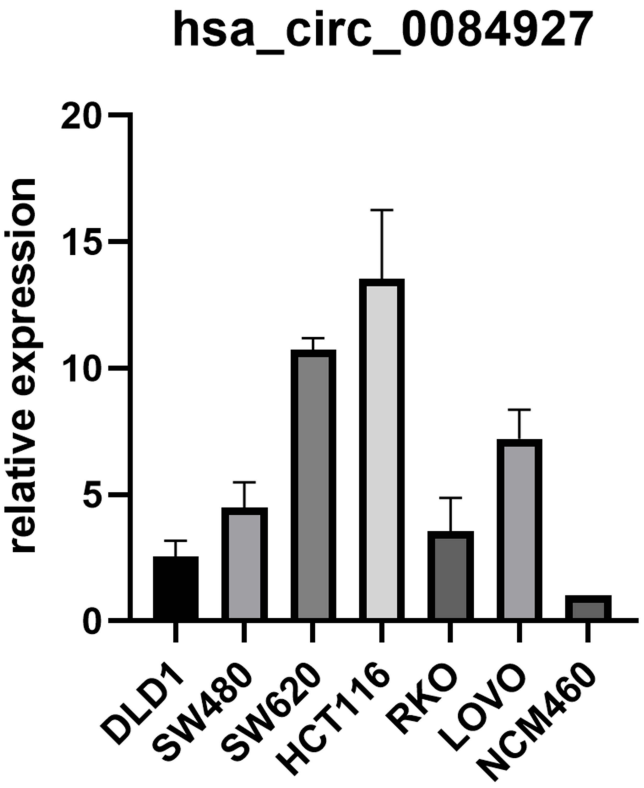

Figure 1 Hsa_circ_0084927 expression in 6 COAD cell lines. Its expression in HCT116, SW620 and LOVO is significantly higher than that in SW480, DLD1, and RKO.

CircRNA-miRNA-mRNA Network Prediction and Analysis

We use Cancer-Specific CircRNAs Database (CSCD) to understand the structural model of the hsa_circ_0084927. Circbank is used to predict sponge miRNA of hsa_circ_0084927. OncomiR and miRCancer were applied to identify differentially expressed miRNAs (DEmiRNAs) in COAD tissue. The intersection of miRNA predicted in Circbank, OncomiR (16) and miRCancer is thought to be the target molecule of hsa_circ_0084927 in COAD. The prediction of target candidates of selected miRNAs was performed using miRWalk3.0 (http://mirwalk.umm.uni-heidelberg.de/). Functional enrichment analysis was conducted on all predicted target genes with miRWalk 3.0 (the newest version). Finally, use Cytoscape software 3.7.2 to visualize the RNA-miRNA-mRNA network.

GO and KEGG Feature Enrichment Analysis

To elucidate the function and regulatory model of miRNAs at the cellular level, analyses including, GO and KEGG analyses of the miRNAs were performed using the clusterProfiler package (R software package, version 4.0.5). The result with $P<0.05$ and count >2 is of significance. DIANA-miR Path (http://diana.cslab.ece.ntua.gr/pathways/) was adopted to perform online gene enrichment analysis of putative targets. Subsequently, KEGG was achieved to analyze the function of target genes.

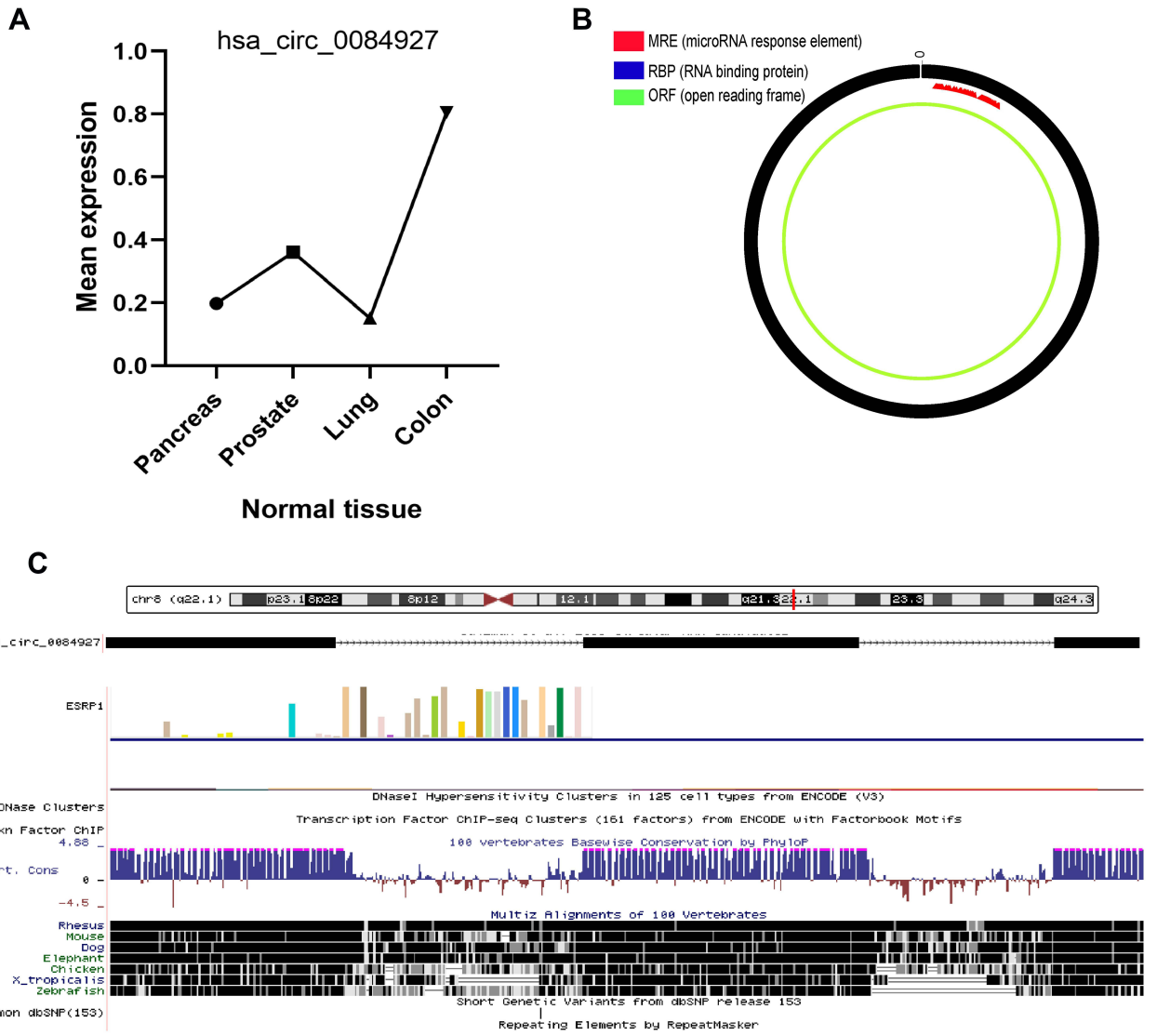

Figure 2 (**A**) The expression of circRNA in normal human tissues in the circAtlas database. (**B**) The hsa_circ_0084927 structure pattern diagram in the CSCD database. (**C**) Schematic diagram of hsa_circ_0084927 locus in ESRP1 gene.

Protein-Protein Interaction (Protein-Protein Interaction, PPI) Network and Hub Gene Construction and Analysis

We have built PPI networks through the String database and Cytoscape software. STRING was a promising and user-friendly online database which may further illustrate the mechanisms between interactive genes. By applying STRING database, PPI network of genes was conducted and an interaction with a combined score > 0.4 was defined as statistical significance. The plug-in Molecular Complex Detection (MCODE) app of Cytoscape (version 3.7.1) is constructed for clustering an interaction network to identify intensively correlated regions.[19] The PPI network was visualized with the app of Cytoscape and the most crucial module in PPI network was obtained using MCODE with the following criteria: node score cutoff = 0.2, degree cutoff =2, k-core = 2 and max depth = 100. According to the node degree, the top 10 genes are listed as hub genes.

Gene Set Enrichment Analysis, GSEA

The high and low expression of 10 Hub genes were grouped in the TCGA database. The hub genes were grouped according to the median expression value respectively and GO-KEGG analysis are performed. Using GSEA 4.0.3 software to assess potential biological functions that may be involved.

Statistical Analysis

Statistical analysis was performed using SPSS 23.0 statistical software, and AI was used for drawing. The relationship between hsa_circ_0084927 and clinicopathological characteristics was tested by chi-square test. Cox regression model is used for single factor and multivariate analysis. Use Kaplan-Meier method to draw OS and DFS curves, and use Log rank test to evaluate their statistical significance. Receiver operator characteristic (ROC) analysis and De long test was constructed to obtain diagnostic utility of targets. It is worth noting that bioinformatics analysis is mainly done using bioinformatics tools and R version 4.0.5. |log2 Fold |> 1 and P<0.05 mRNA or miRNA are considered statistically significant.

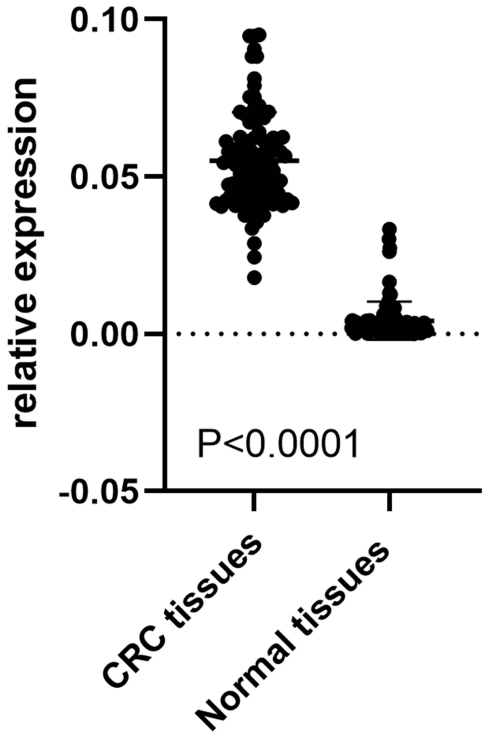

Figure 3 Expression of hsa_circ_0084927 in COAD and adjacent normal tissues.

Results

High-Throughput Sequencing Analysis

Based on the sequencing results, 10 circRNA molecules were expressed differently in the COAD cell line (Supplementary Table 1). Through PCR verification, we found that hsa_circ_0084927 expressed differently in 6 colon cancer cell lines, and showed a steady trend of high expression compared to normal epithelial cell lines (HCT116, SW620 and LOVO are cell lines with high metastatic potential; SW480, RKO and DLD1 are cell lines with low metastatic potential). And the expression of hsa_circ_0084927 in HCT116, SW620 and LOVO is significantly higher than that in SW480, DLD1 and RKO ($P<0.05$, Figure 1). This suggests that hsa_circ_0084927 may be closely related to the transfer and progress of COAD.

Characteristics of hsa_circ_0084927

In the circAtlas database, we found that the expression of hsa_circ_0084927 in various normal tissues is significantly different. Compared with prostate tissue, pancreas, and lung tissues, it is highly expressed in colon tissue (Figure 2A). It shows that hsa_circ_0084927 can be commonly expressed in various tissues, and its high expression in colon tissue may indicate that it participates in some important signal pathways. Through database analysis, the results show that the

Table 1 The Relationship of hsa_circ_0084927 Expression and Clinicopathological Parameters in Patients with Colon Cancer

		n	CircRNA Expression		P	95% CI
			Low	High		
Tumor location	Ascending	52	25	25	0.451	−0.218–0.497
	Descending	26	15	13		
	Transverse	18	8	10		
Gender	Male	47	20	27	0.156	−0.348–0.057
	Female	49	28	21		
Age (year)	≤60	77	38	39	0.800	−0.184–0.142
	>60	19	10	9		
Clinical stage	I–II	29	21	8	0.004	0.091–0.451
	III–IV	67	27	40		
Depth of invasion	Tis-T2	13	2	11	0.007	−0.323–0.052
	T3-T4	83	46	37		
Lymph node metastasis	N0	27	17	10	0.115	−0.036–0.328
	N1-N2	69	31	38		
Distant metastasis	Without	65	37	28	0.050	0.000–0.375
	With	31	11	20		
Differentiation degree	Low	15	7	8	0.484	−0.319–0.152
	Moderate	64	31	33		
	High	17	10	7		
Gross type	Ulceration	59	26	33	0.052	−0.502–0.002
	Elevated	30	16	14		
	Infiltrative	7	6	1		
Cell type	Adenocarcinoma	90	44	46	0.404	−0.140–0.057
	Other	6	4	2		
Perineural invasion	Without	80	42	38	0.278	−0.068–0.235
	With	16	6	10		
Vascular invasion	Without	77	44	33	0.005	0.072–0.386
	With	19	4	15		
Tumor size (cm)	≤5	38	18	20	0.680	−0.242–0.158
	>5	58	30	28		
Preoperative CEA (ng/mL)	≤5	23	13	10	0.478	−0.112–0.237
	>5	73	35	38		

Table 2 Prognosis Analysis of 96 Patients with Colon Cancer

	Univariate Analysis			Multivariate Analysis		
	P	HR	95% CI	P	HR	95% CI
Tumor sites	0.919	0.95	0.351–2.57			
Gender	0.793	0.88	0.339–2.281			
Age	0.166	2.104	0.735–6.022			
Clinical stage	**0.044**	7.976	1.056–60.211	0.215	3.687	0.469–28.956
Depth of invasion	**0.020**	0.289	0.101–0.825	0.686	0.799	0.269–2.372
Lymphatic metastasis	0.261	2.046	0.587–7.131			
Distant metastasis	**0.006**	3.873	1.471–10.199	0.243	1.822	0.665–4.992
Tumor differentiation.	0.355	0.448	0.082–2.451			
Gross type	0.212	0.452	0.130–1.573			
Cell type	0.213	2.560	0.584–11.227			
Perineural invasion	**0.003**	4.254	1.616–11.197	**0.022**	3.301	1.186–9.187
Vascular invasion	0.462	1.480	0.521–4.206			
Tumor size	0.906	1.060	0.403–2.787			
Preoperative CEA	0.058	7.071	0.934–53.538			
hsa_circ_0084927 expression	**0.007**	5.512	1.582–19.205	**0.037**	3.839	1.086–13.563

Note: Bold indicates a P value less than 0.05.

hsa_circ_0084927 is made up of the inclusions of the ESRP1 gene, located at chr8:95676924–95677424, resulting in a mature sequence of 287 nt in length (Figure 2B and C). The CSCD shows that hsa_circ_0084927 contains structural reaction elements (microRNA response element, MREs) that interact with miRNA (miR-106 family, miR-1262, etc.), suggesting that hsa_circ_0084927 can regulate the development of COAD by acting on key miRNAs of COAD.

Relationship Between hsa_circ_0084927 Expression and Clinical Characteristics of COAD Patients

Depending on the median expression of the hsa_circ_0084927 in the COAD tissue, COAD patients can be divided into high and low expression groups. The relationship hsa_circ_0084927 between high and low expression group and clinic-pathological characteristics of COAD patients was analyzed by Chi-square Test. The results showed that the expression of circRNA in cancer tissue was significantly higher than that in adjacent normal tissues (Figure 3); Analysis of clinicopathological data showed that the level of hsa_circ_0084927 expression was associated with clinical stage, depth of immersion and vascular invasion (P<0.05, Table 1).

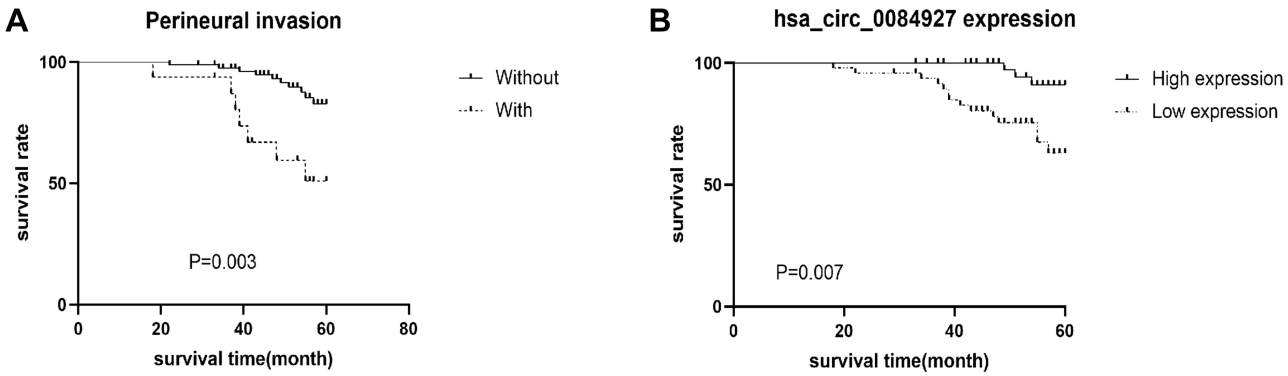

Figure 4 The expression of hsa_circ_0084927 (**A**) and perineural invasion (**B**) are related to OS in COAD patients.

Table 3 The Expression Level of Hsa_circ_0084927 in the Plasma of COAD Patients and Its Baseline Characteristic

		n	Circ RNA Expression	P value
Gender	Male	22	3.93±0.93	0.89
	Female	8	3.98±0.92	
Age	≤60	15	3.69±0.69	0.13
	>60	15	4.20±1.05	
Clinical stage	I–II	20	3.81±0.95	**0.02**
	III–IV	10	4.49±0.63	
T stage	Tis-T2	8	3.42±0.73	**0.04**
	T3-T4	22	4.13±0.92	
N stage	N0	20	3.71±0.95	**0.02**
	N1-N2	10	4.42±0.63	
M stage	Without	15	3.80±1.12	0.41
	With	15	4.11±0.66	
Differentiation	Low	6	3.98±0.52	0.88
	High	24	3.93±0.99	
Tumor size (cm)	≤5	20	4.02±0.98	0.53
	>5	10	3.81±0.79	
CEA (ng/mL)	≤5	17	3.91±0.87	0.78
	>5	13	4.01±0.99	

Note: Bold indicates a P value less than 0.05.

Relationship Between hsa_circ_0084927 Expression Levels and OS in COAD Patients

The univariate and multivariate analysis COX regression analysis of the above clinical parameters was carried out. The univariate analysis showed that clinical stage, immersion depth, distant metastasis, perineural invasion, and hsa_circ_0084927 were related to OS, and further multivariate analysis showed that perineural invasion and high expression of hsa_circ_0084927 were independent risk factors for poor prognosis in COAD patients (Table 2, Figure 4). KM analysis showed that the OS in the high hsa_circ_0084927 expression group was significantly lower than that of the low expression group (53.879m and 59.233m, respectively). This indicates that the higher the expression of hsa_circ_0084927 in the COAD tissue, the worse the prognosis for COAD patients.

Diagnostic Efficacy of Plasma hsa_circ_0084927

This study further investigated the plasma hsa_circ_0084927 expression in COAD patients and healthy subjects. Meanwhile, qRT-PCR was applied to detect the hsa_circ_0084927 expression in 30 COAD patients and 30 healthy subjects (Table 3). The expression of plasma hsa_circ_0084927 was correlated with T stage, N stage and clinical stage ($P<0.05$). The results showed that plasma hsa_circ_0084927 expression was significantly up-regulated in COAD patients compared with healthy subjects. The hsa_circ_0084927 expression in preoperative plasma was significantly higher than that in postoperative plasma ($P < 0.05$). Next, ROC curve analysis was performed to evaluate the diagnostic efficacy of plasma hsa_circ_0084927. The results showed that preoperative plasma hsa_circ_0084927 (AUC=0.78, 95% CI:0.65–0.87) was superior to CEA (AUC=0.66, 95% CI:0.53–0.78) and was a better diagnostic marker for COAD patients (AUC, $P < 0.05$, Figure 5).

The Effect of hsa_circ_0084927-Knockdown on the Proliferation of COAD Cells

We transferred the designed siRNA fragments into 6 cell lines, and selected cell lines with a transfection efficiency of more than 80% for the next step. After transfecting the HCT116, and LOVO, CCK-8 experiments were used to detect their proliferation capacity. The results of CCK-8 experiment showed that after continuous observation of 96h, the OD450 values of the experimental group were 2.25±0.006 (HCT116), 2.217±0.02 (LOVO) respectively, the

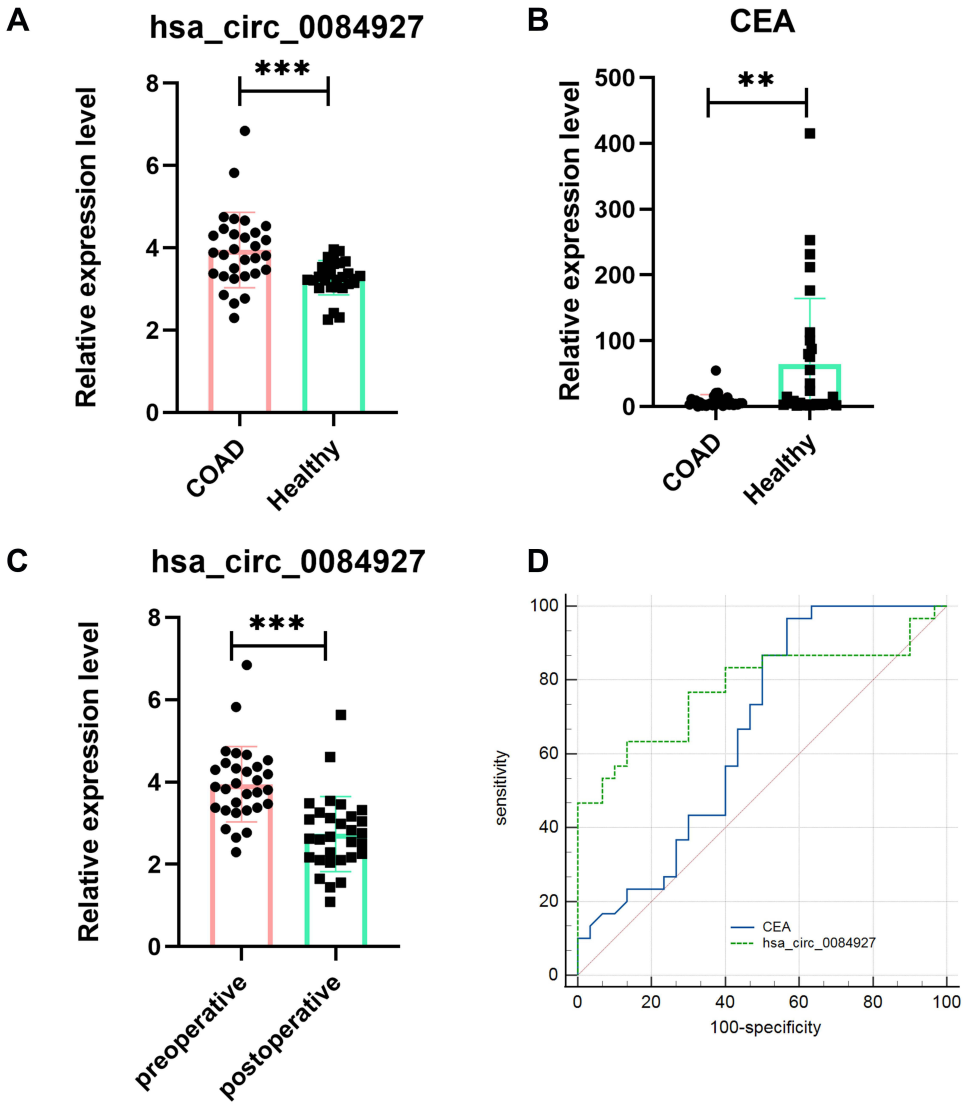

Figure 5 Expression of plasma hsa_circ_0084927 and CEA. (**A**) There is a difference in the expression of plasma hsa_circ_0084927 between COAD patients and healthy subjects (3.95±0.91 VS 3.27±0.42). (**B**) CEA was differentially expressed between COAD patients and normal people (7.82±10.4 VS 64.71±99.76). (**C**) The preoperative hsa_circ_0084927 expression in the plasma of COAD was higher than its postoperative expression level (3.95±0.91 VS 2.74±0.16). (**D**) ROC curve analysis shows that plasma RNA is superior than traditional CEA in the identification of COAD patients. *P<0.05, **P<0.01, ***P<0.001.

corresponding control group D450 value is 1.81±0.02, 1.65±0.03 respectively (Figure 4A), indicating that the knockdown of hsa_circ_0084927 significantly inhibited the proliferation of these three COAD cell lines (Figure 6).

The Effect of hsa_circ_0084927-Knockdown on the Migration of Colon Cancer Cells

After cell transfection, the effect of hsa_circ_0084927 on migration was detected by the scratch experiment and transwell chamber migration experiment. Scratch test results showed that after transfection with Si-Circ, the cell mobility (%) of the experimental group was 0.31±0.01 (HCT116) and 0.24±0.02 (LOVO), and that of the control group was 0.38±0.02 (HCT116) and 0.35±0.01 (LOVO), respectively. These results suggest that down-regulation of hsa_circ_0084927 can significantly inhibit the horizontal migration of COAD cells (Figure 7).

The results of transwell chamber migration experiments showed that the cell numbers of HCT116 and LOVO in control groups were 86.5±5.2 /field of view and 65.8±3.9/field of view, respectively. The cell number in the experimental group was 38.3±6.2/field, (29.7±5.4) /field, indicating that the knockdown of hsa_circ_0084927 significantly inhibited

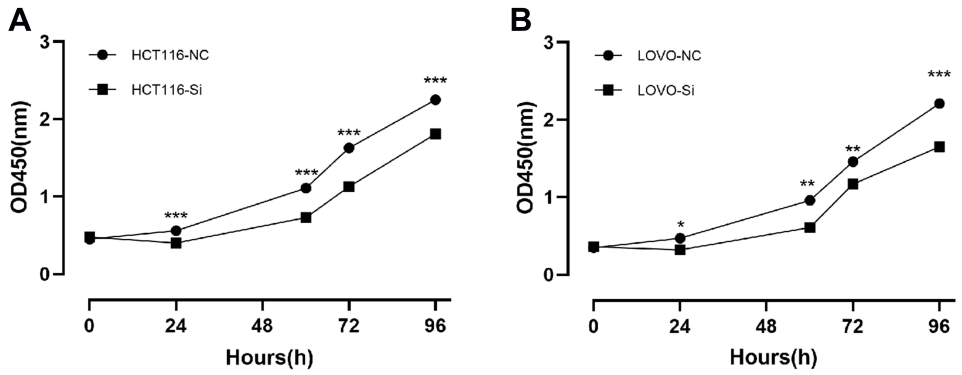

Figure 6 SiRNA knockdown of hsa_circ_0084927 significantly inhibited the proliferation ability of COAD cell lines. (**A**) After hsa_circ_0084927 knockdown, the proliferation ability of HCT116 cells was significantly down-regulated; The circle represents the negative control of HCT116 knockdown group; The square represents the HCT116 knockdown group; (**B**) After hsa_circ_0084927 knockdown, the proliferation ability of LOVO cells was significantly down-regulated; The circle represents the negative control of LOVO knockdown group; The square represents the LOVO knockdown group. **means P<0.01, ***means P< 0.001.

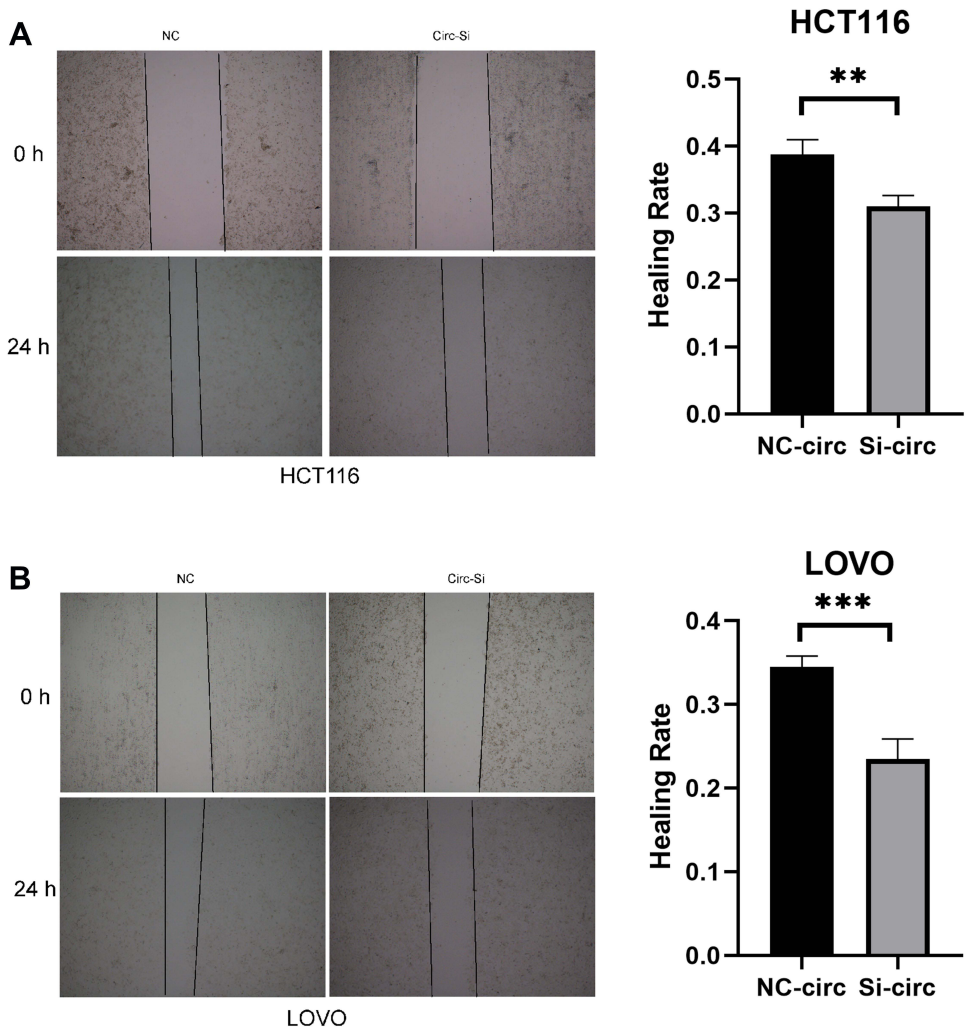

Figure 7 (**A**) Scratch test showed that silencing hsa_circ_0084927 inhibited horizontal migration of HCT116 cells. (**B**) Scratch test showed that silencing hsa_circ_0084927 inhibited horizontal migration of LOVO cells. **P<0.01, ***<0.001.

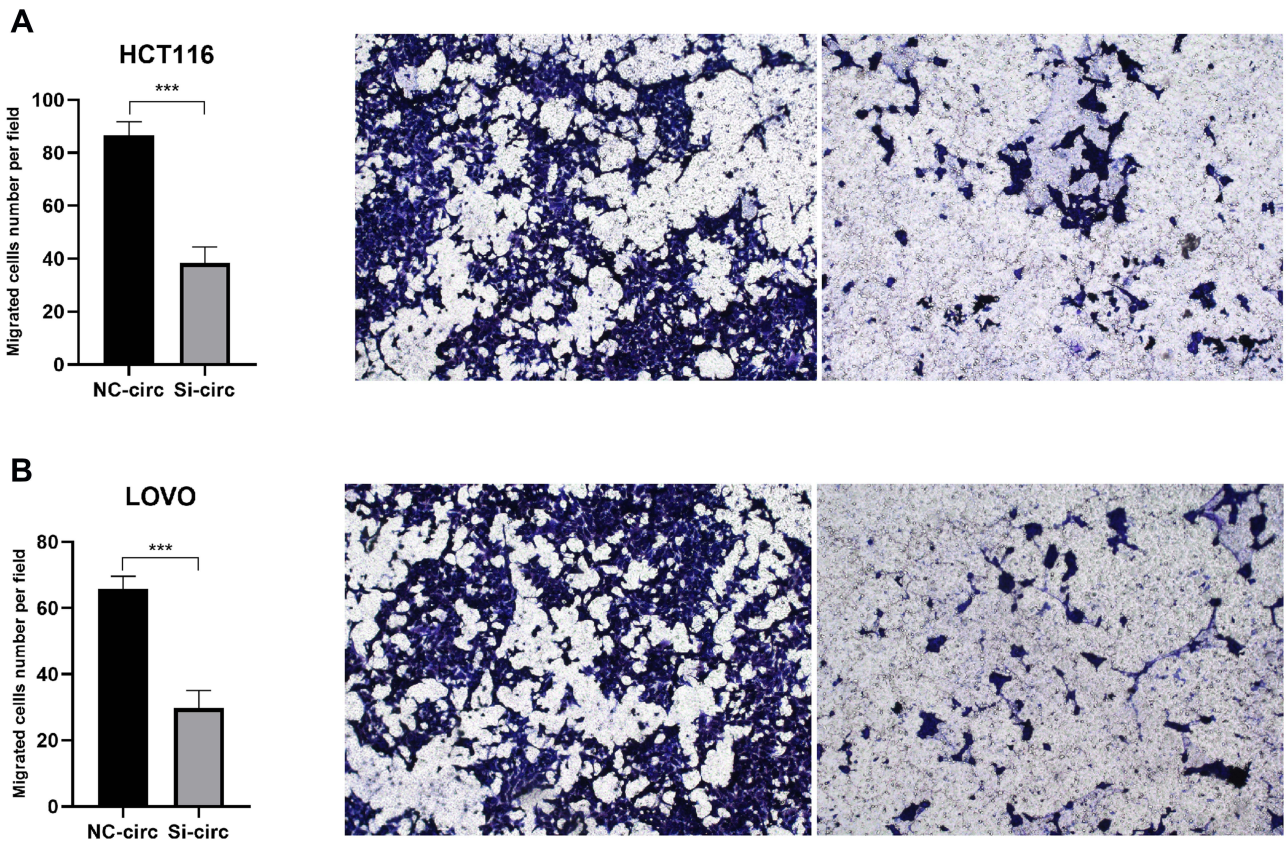

Figure 8 Migration experiment after hsa_circ_0084927 knockdown. (**A**) Knockdown of hsa_circ_0084927 can significantly inhibit the vertical migration ability of HCT116 cells. (**B**) Knockdown of hsa_circ_0084927 can significantly inhibit the vertical migration ability of LOVO cells. ***P<0.001.

the migration capacity of these two COAD cells (Figure 8). The results of the scratch test and transwell migration experiments confirmed each other.

The Effect of hsa_circ_0084927 Knockdown on the Invasion Ability of Colon Cancer Cells

After Si-NC and Si-Circ were transfected into HCT116 and LOVO cells, their invasion ability was tested by transwell cell invasion test. The results of the transwell cell invasion experiment showed that the number of cells in the control group of HCT116 and LOVO cells was (68.0±4.9)/field and 51.4±3.9/field, respectively, and the number of cells in the experimental group was (21±2.4)/field and (18.4±1.2)/field. The results show that the knockdown of hsa_circ_0084927 can significantly inhibit the invasion ability of colon cancer cells (Figure 9).

Prediction of miRNA

Taking the intersection of the miRNA in the oncomiR, miRcancer and circBank database, we obtained the differentially down-regulated miRNA: hsa-miR-106b-5p (Figure 10A). Next, miRTarbase and targetscan were used to predict target genes regulated by hsa-miR-106b-5p, and a total of 134 related mRNAs were obtained. Finally, based on the circRNA-miRNA-mRNA regulatory network, we used Cytoscape 3.3.1 to visualize the ceRNA network (Figure 10B and C, Supplementary Table 2).

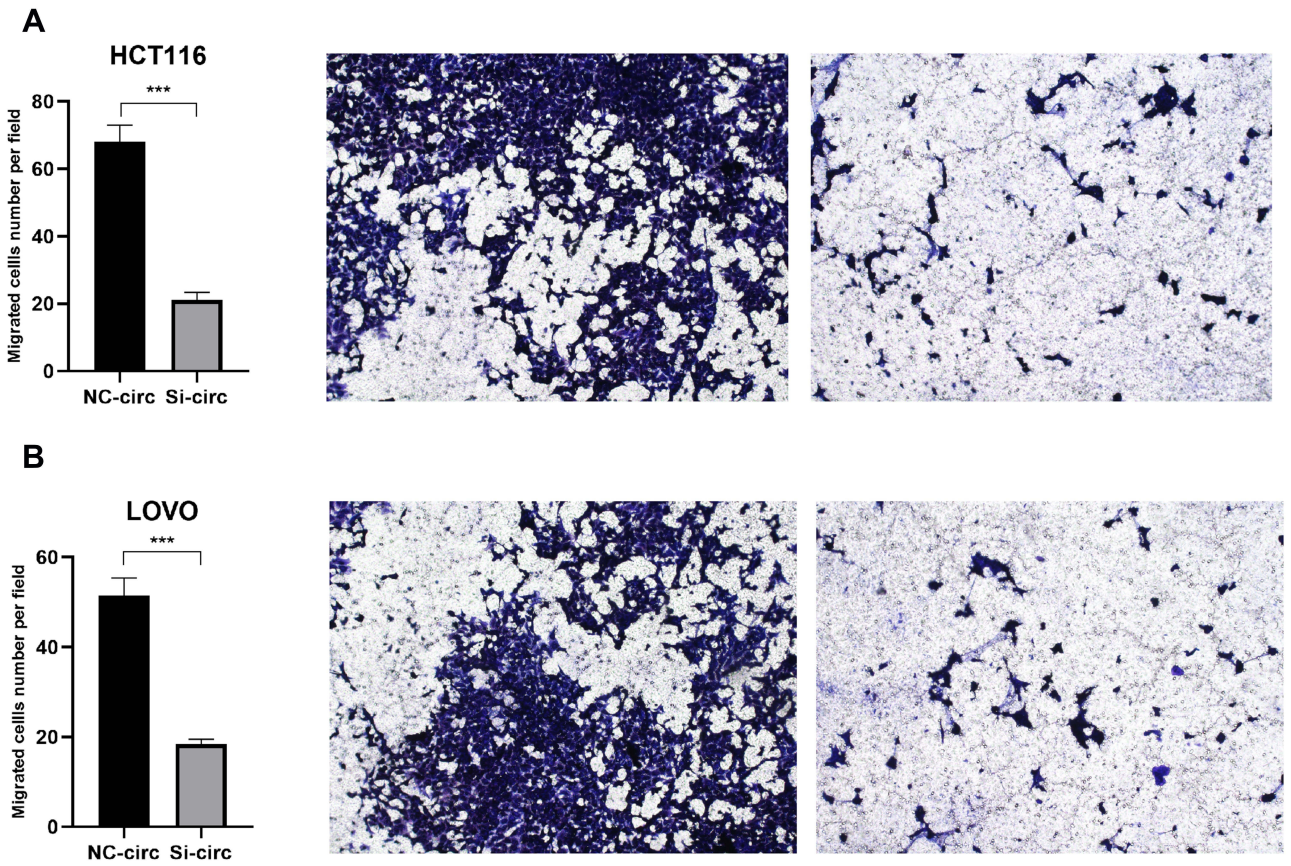

Figure 9 Invasion experiment after hsa_circ_0084927 knockdown. (**A**) Knockdown of hsa_circ_0084927 can significantly inhibit the invasion ability of HCT116 cells. (**B**) Knockdown of hsa_circ_0084927 can significantly inhibit the invasion ability of LOVO cells. ***P<0.001.

mRNA Function Analysis

To elucidate the potential biological functions of hsa_circ_0084927 in the development of COAD, GO (Figure 11A–C) and KEGG (Figure 11D) enrichment analyses were performed on 134 target genes in established ceRNA networks. GO bio-process analysis showed that the target genes were involved in Golgi vesicle transport, endomembrane system organization, and cytosolic transport. For cell composition (CC) analysis, the target genes are significantly rich in trans-Golgi network and clathrin-coated vesicle. In addition, molecular functional analysis of these mRNAs includes proximal promoter sequence-specific DNA binding, RNA polymerase II proximal promoter sequence-specific DNA, and GTPase binding. Besides, KEGG's enrichment results show that DEmRNAs are significantly associated with a variety of cancer-related pathways, such as cell cycle, colon cancer, FOXO signaling pathway, Wnt signaling pathway, pathway in cancer, non-small cell lung cancer and other networks.

Using the STRING database to analyze the relationship between 134 mRNAs in the ceRNA network, and Cytoscape 3.7.2 is used to visualize the PPI network. We used the CytoHubba plug-in to identify the top 10 hub genes, including OCRL, RBBP7, MECP2, ITCH, CREB1, FYN, LDLR, TWIST1, STAT3, VEGFA (Figure 12A and B), and also extracted the more important models (Figure 12C–E).

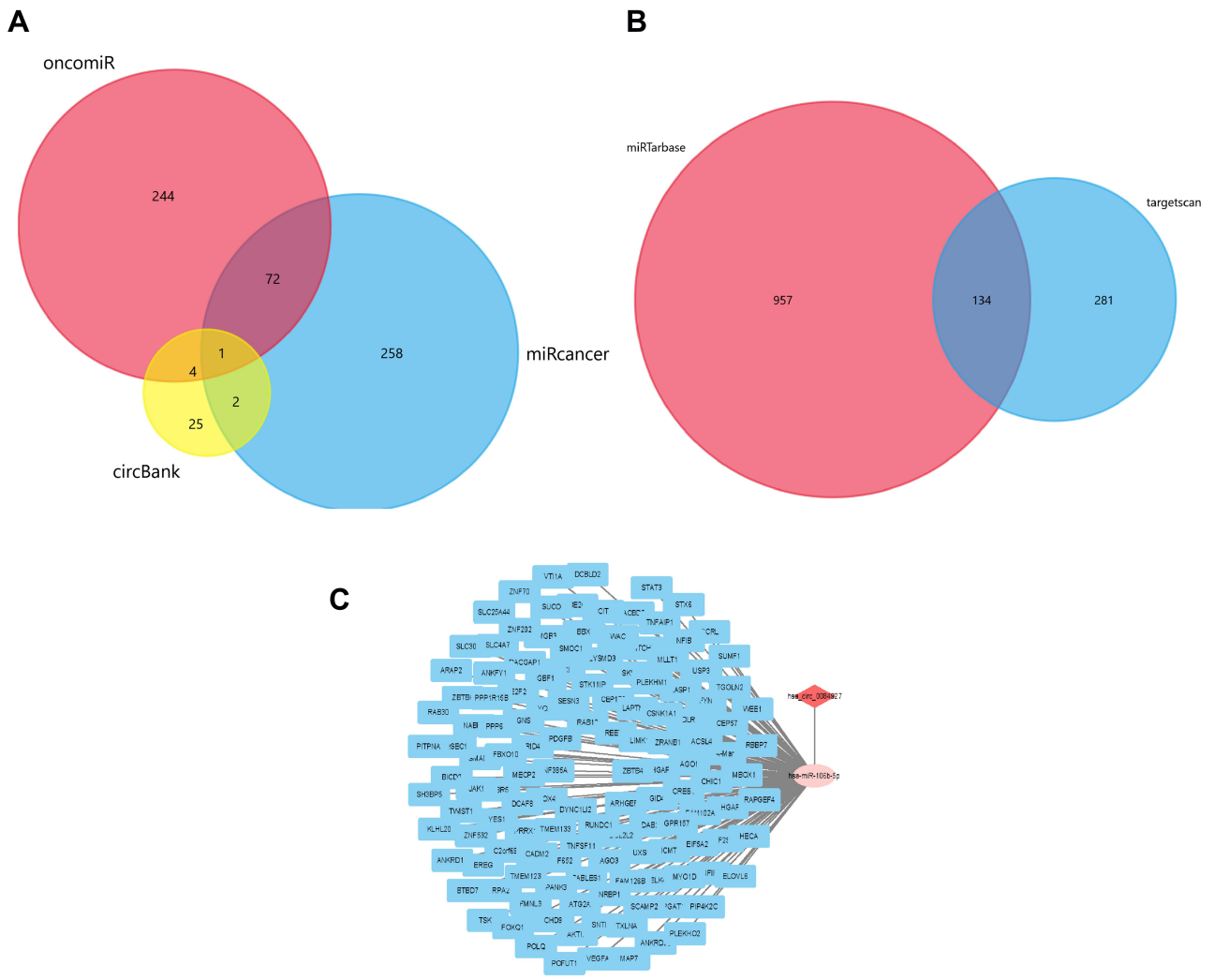

Figure 10 Construction of the ceRNA network of hsa_circ_0084927 in COAD. (**A**) Using databases to predict the sites that may bind to hsa_circ_0084927, and hsa-miR -106b-5p is obtained after the intersection of 3 databases. (**B**) The 134 target genes that may be regulated by hsa-miR-106b-5p are predicted and obtained through the databases. (**C**) Visualize the ceRNA network with Cytoscape software.

Identification of Hub Genes and Construction of has_circ_0084927/miR-106b-5p/VEGFA

To learn more about the biological function of these 10 hub genes in COAD, we used the Oncomine database to detect the expression of the genes in COAD. The results showed that the expression level of the hub genes in COAD tissue was significantly higher than that of normal tissues (Figure 13). In addition, the Human Protein Altas Database and immunohistochemistry (IHC) showed differences between OCRL, RBBP7, MECP2, ITCH, CREB1, FYN, LDLR, TWIST1, STAT3 and VEGFA in the actual expression of normal colonic tissue and COAD (Figure 13). Patient data for IHC are shown in Table 4. The effects of these hub genes on the prognosis of COAD are then assessed using the GEPIA database. We found that VEGFA patients had shorter RFS, and patients with high level MECP2 and ITCH had shorter OS (Figures 14 and 15, $P<0.05$). We then verified the correlation between has_circ_0084927, miR-106b-5p and the three hub genes by PCR, and found that only VEGFA showed significant correlation with has_circ_0084927 and miR-106b-5p in LOVO cell lines (Figure 16).

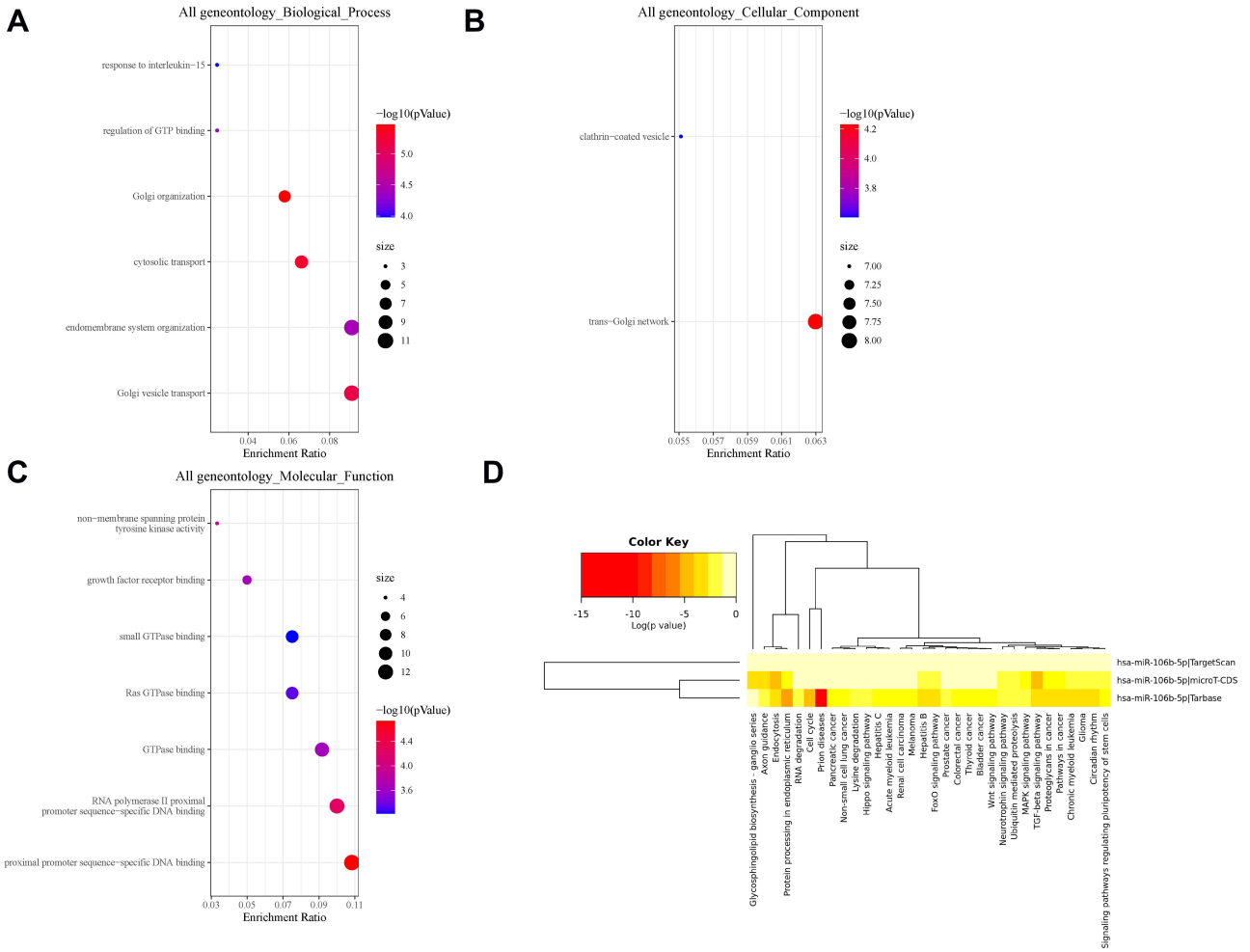

Figure 11 Bioinformatics analysis of target genes regulated by HSA_CIRC_0084927. (**A**–**C**), GO function analysis of target genes; (**D**) KEGG signaling pathway analysis of target genes. The construction of PPI networks and hub genes.

GSEA

To further determine the possible pathways and mechanisms of these three hub genes in the COAD process, we used the TCGA database for GSEA analysis. The results showed that ITCH, MECP2 and VEGFA were mainly enriched in cell cycle, apoptosis, adherens junction, Wnt pathway, mismatch repair, P53 pathway, colorectal cancer, etc. Therefore, the circular RNA may regulate cell apoptosis and cell cycle through the ceRNA network, thereby affecting tumor progression (see Supplementary Tables 3–8).

Discussion

CircRNA is involved in the proliferation, invasion, metastasis, and apoptosis of COAD cells. Zhang et al[19] using RNA-seq technology found that the overall circRNA was significantly less expressive in colorectal cancer tissue and cell lines. Compared to paracancer tissues, 125 circRNA expressions in colorectal cancer tissue were reduced, and 76 circRNA expressions were increased.[19] For example, hsa_circRNA_103809 is low expressed in colorectal cancer tissue and associated with distant metastasis.[19,20] Hsa_circ_0084927 is differentially expressed in clear cell renal cell carcinoma,[21] and it is also significantly up-regulated in lung cancer-related malignant pleural effusion and may be involved in the PI3K-Akt signaling pathway.[22] Hsa_circ_0084927 promotes the progression of CC by up-regulating TPD52 via sponge adsorption of miR-634, which provides new evidence for its use as a promising therapeutic target.[23] The significantly

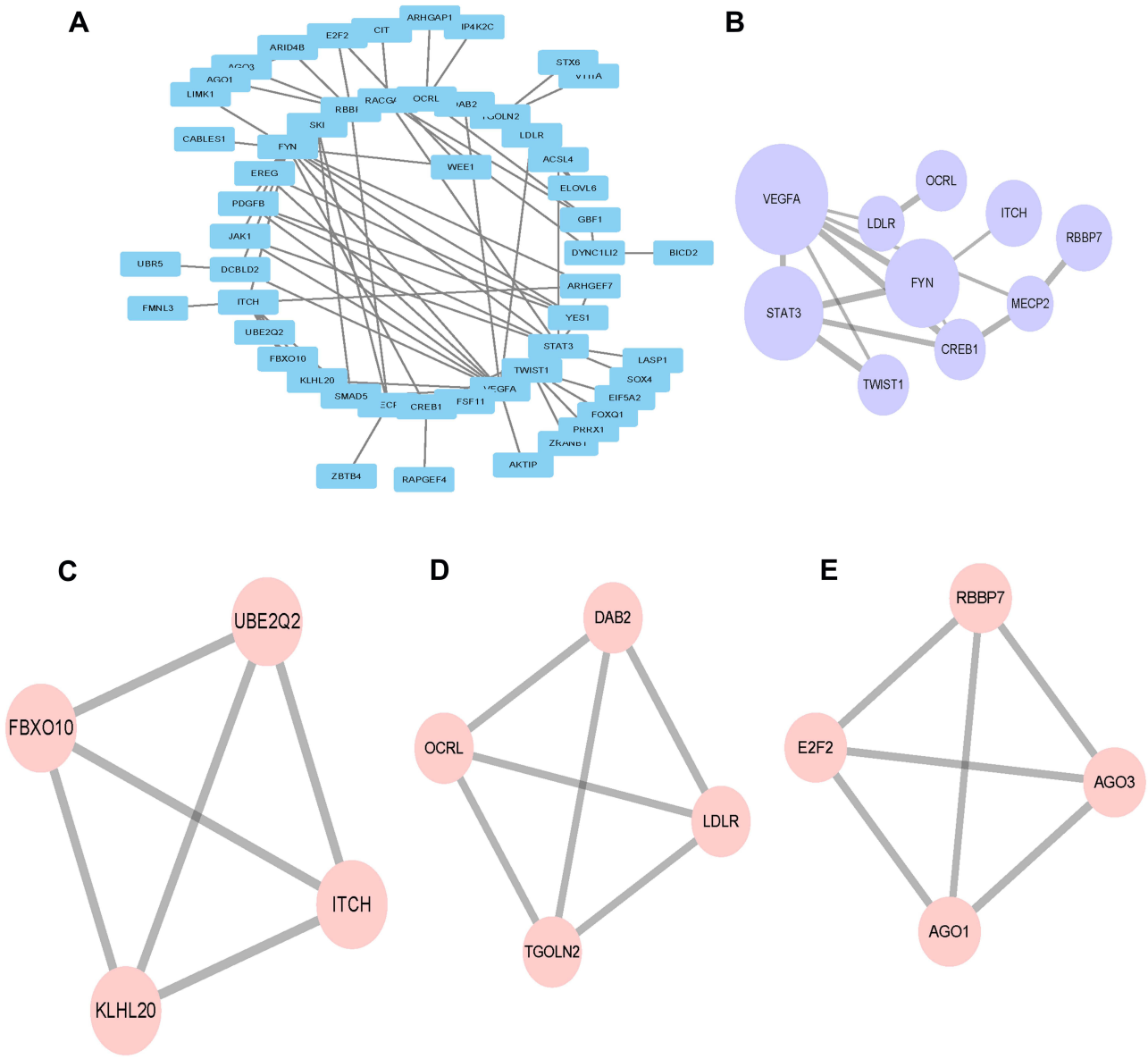

Figure 12 PPI network construction of target genes. (**A**) PPI map of 134 genes. (**B**) Interaction diagrams of top 10 hub genes were extracted. (**C–E**) Important modules extracted from the hub genes.

high expression in these tumors suggests that they may have potential cancer-promoting possibilities, which are worthy of further study. Up to now, there is very little research on hsa_circ_0084927, and more importantly, the function and mechanism of hsa_circ_0084927 have not been fully studied, and its role in tumor is unclear. Therefore, the study of hsa_circ_0084927 in COAD is of great significance. Based on sequencing data and preliminary experimental verification, our study suggested that hsa_circ_0084927 was significantly overexpressed in COAD tissue and cell lines, affecting biological behavior and prognosis, and plasma hsa_circ_0084927 can serve as a potential noninvasive biomarker in COAD.

One of the mechanisms of CircRNA is that it competitively binds with miRNA to regulate target genes. Reconfirmed by the miRCancer database, hsa-miR-106b-5p is reduced in colorectal cancer cells and can regulate downstream pathways to promote cancer development;[24] Studies have shown that hsa-miR-106b-5p is differently expressed in liver

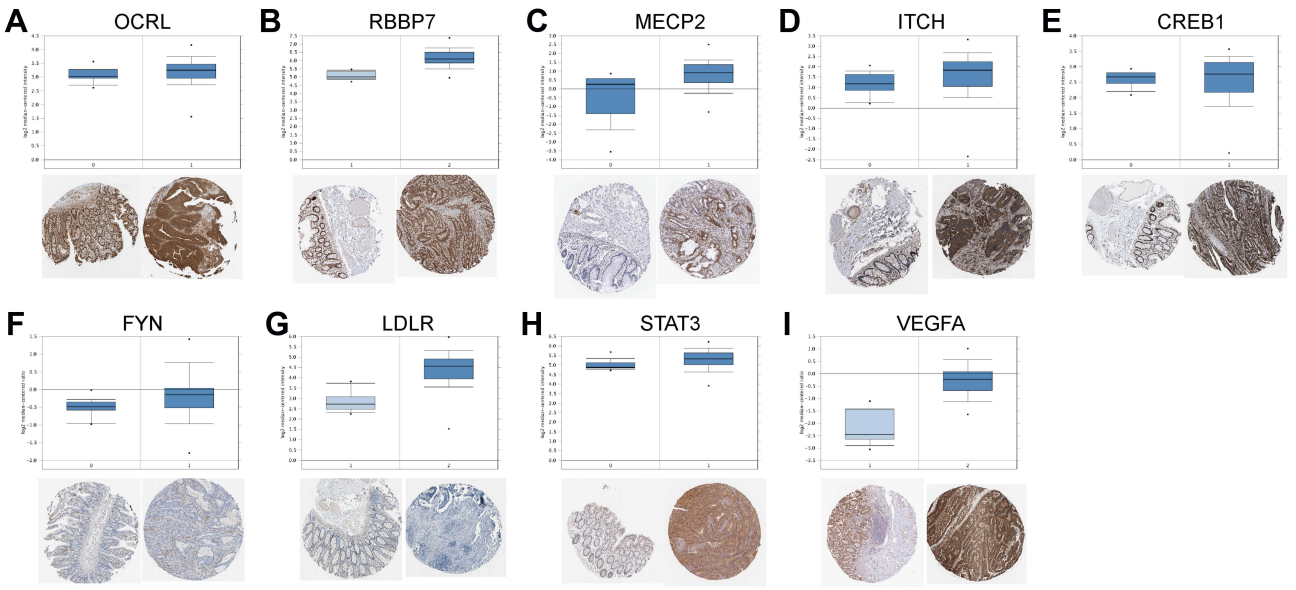

Figure 13 The expression of hub gene at transcription level and post-transcription level was verified by Oncomine database and HPA database. The left side of each subgraph represents paracancer tissue and the right side represents cancer tissue. (**A**) OCRL, (**B**) RBBP7, (**C**) MECP2, (**D**) ITCH, (**E**) CREB1, (**F**) FYN, (**G**) LDLR, (**H**) STAT3, (**I**) VEGFA.

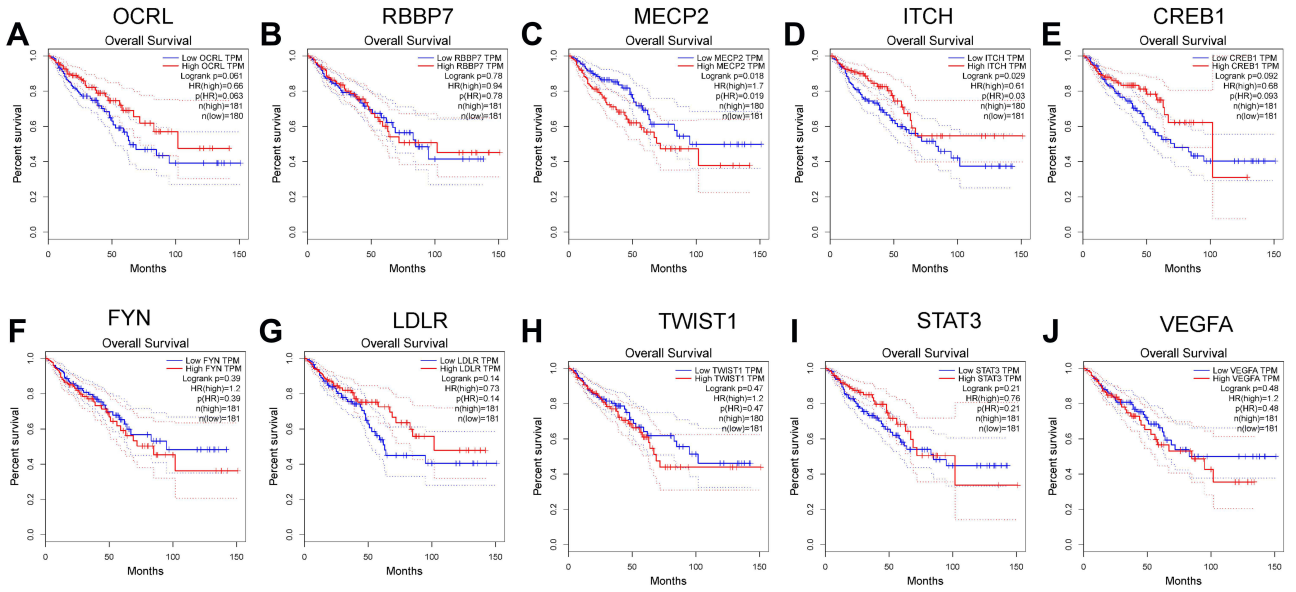

Figure 14 Relationship between the expression of 10 hub genes and OS in COAD. (**A**) OCRL, (**B**) RBBP7, (**C**) MECP2, (**D**) ITCH, (**E**) CREB1, (**F**) FYN, (**G**) LDLR, (**H**) TWIST1, (**I**) STAT3, (**J**) VEGFA.

and breast cancer, but its function and mechanism have not been thoroughly clarified.[25,26] Compared with stage I–II, miR-106b is significantly down-regulated in colorectal cancer tissues at stage III–IV, inhibiting the migration and invasion of COAD cells;[27] in addition, miR-106b-5p can also inhibit the metastasis of endometrial and breast cancer;[28,29] In the Indonesian population, it can be used as a liquid biomarker for prostate cancer;[30] In the OncomiR database, we found that its expression in colon tissue is higher than that in cancer tissue, but it is of no value in terms of stage and prognosis.

Table 4 Clinical Baseline Features of Immunohistochemistry in Human Protein Atlas Database

Gene Symbol	Tissue Type	ID	Gender	Age	Staining	RNA Consistency*
VEGFA	Endothelial cells	1985	Female	79	Low	Medium
	Tumor cells	2931	Male	77	High	
STAT3	Endothelial cells	1962	Male	83	Low	Medium
	Tumor cells	5003	Female	87	Medium	
LDLR	Endothelial cells	1506	Female	55	Low	Low
	Tumor cells	3408	Male	63	Low	
FYN	Endothelial cells	1454	Female	46	Low	Low
	Tumor cells	3074	Male	72	High	
OCRL	Endothelial cells	1506	Female	55	High	Medium
	Tumor cells	3074	Male	72	High	
CREB1	Endothelial cells	1423	Female	56	High	High
	Tumor cells	2096	Male	72	High	
ITCH	Endothelial cells	1423	Female	56	Medium	Medium
	Tumor cells	3274	Female	89	High	
MECP2	Endothelial cells	177	Male	1	Medium	High
	Tumor cells	55	Female	81	High	
RBBP7	Endothelial cells	3472	Male	26	High	High
	Tumor cells	4606	Female	48	High	

Note: *RNA consistency between antibody staining and RNA expression data, represent data reliability.

Pathway enrichment analysis showed that the 134 genes were closely related to the Wnt signaling pathway, cell cycle, and colon cancer pathways. The Wnt signaling pathway plays an important role in the occurrence and development of COAD. The HPA database and immunohistochemistry showed that the expression of these 10 genes in COAD was higher than that in adjacent tissues. In this way, we confirmed our above analysis from the protein expression level, laying the foundation for the diagnosis and prognostic research of these 10 hub genes. Analysis of the Gepia database shows that VEGFA, MECP2 and ITCH are related to the prognosis of COAD patients. At the same time, we detected the expression of VEGFA, MECP2, ITCH and miR-106-5p in the three COAD cell lines, and initially verified the rationality

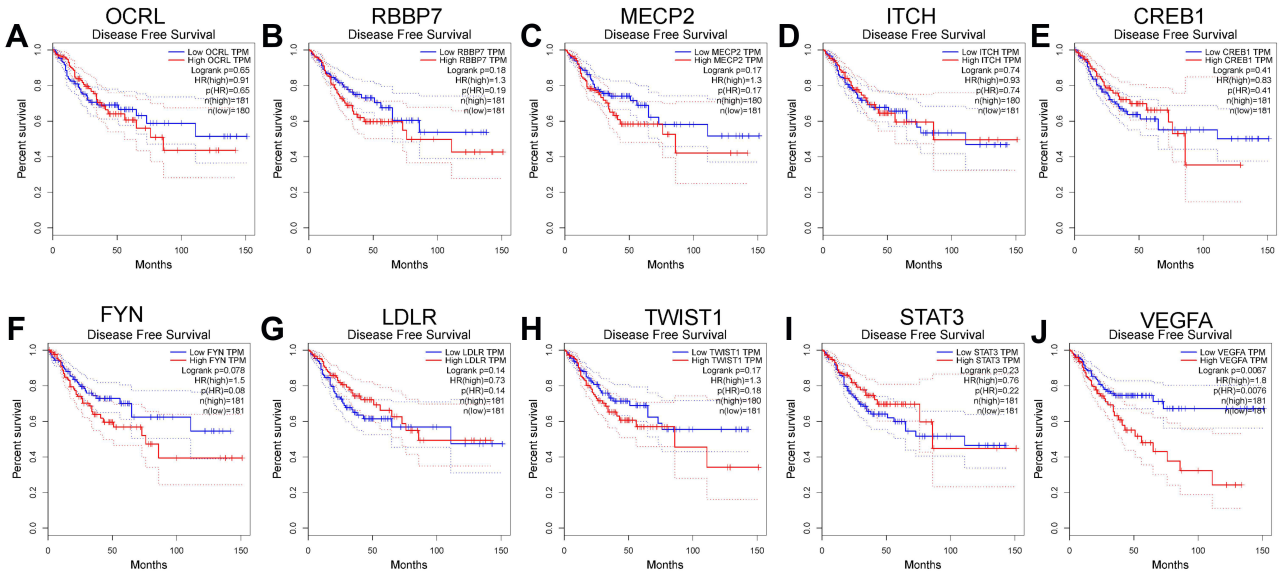

Figure 15 Relationship between DFS and expression of 10 hub genes in COAD. (**A**) OCRL, (**B**) RBBP7, (**C**) MECP2, (**D**) ITCH, (**E**) CREB1, (**F**) FYN, (**G**) LDLR, (**H**) TWIST1, (**I**) STAT3, (**J**) VEGFA.

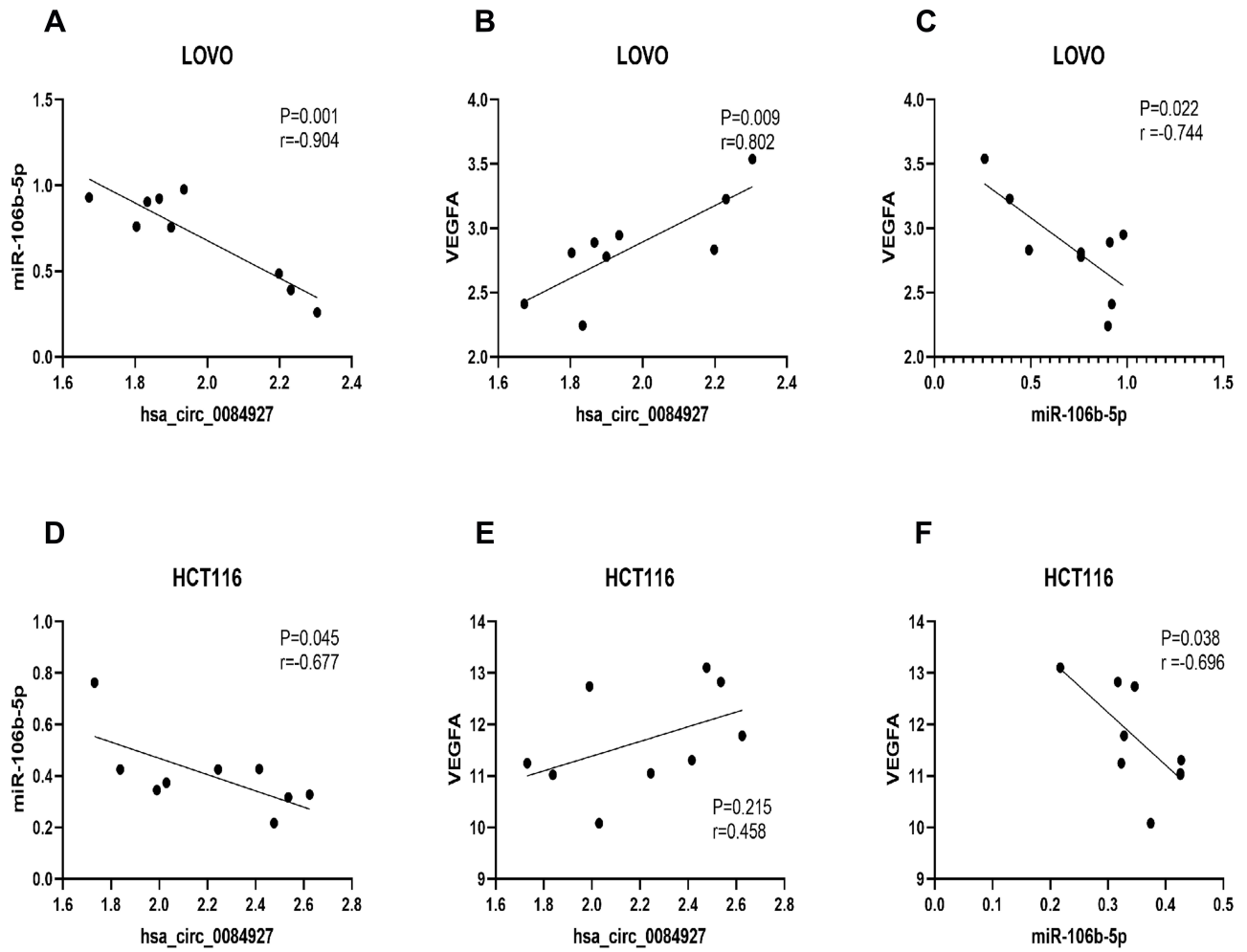

Figure 16 Correlation analysis showed that has_circ_0084927 expression was negatively correlated with miR-106b-5p (**A**) and positively correlated with VEGFA (**B**) expression in LOVO cell lines. The expression of miR-106b-5p was negatively correlated with VEGFA expression (**C**). In HCT116, has_circ_0084927 expression was negatively correlated with miR-106b-5p (**D**), but not with VEGFA (**E**) expression. The expression of miR-106b-5p was negatively correlated with VEGFA (**F**).

of the circRNA-miRNA-mRNA regulatory network. VEGFA can promote tumor invasiveness by inducing epithelial-mesenchymal transition.[31] There is a well-recognized association between high baseline circulating VEGFA levels and poor prognosis for different types of cancer.[32,33] Research shows that ITCH promotes tumor progression in hepatocellular carcinoma, breast cancer, pancreatic cancer and chronic lymphocytic leukemia.[34–36] The previous study indicates that the inhibition of MECP2 function in DLD1 cells leads to a decrease in cell growth, which indicates that MECP2 may be a potential target for the treatment of colorectal cancer, however, its function is far from clear.[37]

The 3 hub genes we extracted were mainly rich in cell cycle, apoptosis, adherens junction, Wnt pathway, mismatch repair, the P53 pathway, colorectal cancer, and other pathways. Cell cycle and apoptosis pathways have been widely reported to have an inestimable effect on tumor occurrence, progression, and prognosis.[38] P53 pathway is abnormal in many tumors and is an important regulator of cell cycle and cell metabolism. Wnt is a critical pathway for regulating EMT.

The cancer-promoting function of hsa_circ_0084927 and its potential value as a therapeutic target can be further confirmed in future in vitro and in vivo studies and clinical trials. For example, directly interfering hsa_circ_0084927 with drugs or designing siRNA delivery to the tumor site to interfere with the expression and function of oncogenic circRNA; using organic or inorganic materials to affect the function of circRNA. Circular RNA, as a ceRNA, is expected to replace anti-nucleotide chemical drugs and regulate the level of miRNA, in order to regulate the upstream and downstream gene networks, and have an impact on human physiology and disease. In

recent years, many studies have reported the application of circular RNAs as potential therapeutic targets. For example, Xu Bin et al[39] designed and synthesized si-ciRS-7 specific for ciRS-7, and successfully prepared PBAE/si-ciRS-7 nanometers Complex. Their study confirms that drug development of PBAE/si-ciRS-7 nanocomplexes targeting ciRS-7 may be a promising therapeutic strategy for renal cell carcinoma. Wang et al demonstrated that targeting ciRS-122 by systemic injection of exosome-delivered siRNA in an oxaliplatin-resistant mouse model of CRC sensitized CRC to oxaliplatin, suggesting a new potential method can reverse oxaliplatin resistance in CRC.[40] Down-regulated circRNAs have been shown to negatively regulate CRC growth and metastasis. Inducing the expression of these tumor suppressor circRNAs by physicochemical factors in CRC cells or tissues may have significant antitumor effects.[41]

This study preliminarily verified that hsa_circ_0084927 could play a tumor-promoting role in COAD, and proposed that it could be used as a non-invasive detection marker for prognostic assessment in plasma and effectively established the hsa_circ_0084927/miR-106b-5p/VEGFA network. In addition, it is also found that VEGFA, MECP2 and ITCH in the network may affect the prognosis of COAD patients by regulating the cell cycle and other ways. However, the specific mechanisms by which hsa_circ_0084927 and ceRNA networks regulate the development of COAD deserve further study.

Abbreviations

COAD, colon adenocarcinoma; ceRNA, competitive endogenous RNA (ceRNA); RT-PCR, real-time quantitative PCR; circRNA, Circular RNA; CSCD, Cancer-Specific CircRNAs Database; DEmiRNAs, differentially expressed miRNAs; PPI, Protein-Protein Interaction; ROC, Receiver operator characteristic.

Data Sharing Statement

The original data supporting the article is included in the article/Supplementary Materials.

Ethics Approval

This study was approved by the Ethics and Human Subject Committee of Guangxi Medical University Cancer Hospital (LW2022020).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. Equal contribution: Yi Chen and Chunrun Ling have contributed equally to this work.

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Disclosure

The authors report no conflicts of interest for this work.

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