Original Article

RNA binding protein CUGBP1 mediates the liver metastasis of colorectal cancer by regulating the ErbB signal pathway

Zhi-Peng Qi[1,2#], Zhang-Han Chen[1,2#], Dong-Li He[3#], Shi-Lun Cai[1,2], Bing Li[1,2], Di Sun[1,2], Zhen-Tao Lv[1,2], En-Pan Xu[1,2], Qiang Shi[1,2,4], Yun-Shi Zhong[1,2,4]^, Jian-Min Xu[5]

[1]Endoscopy Center, Zhongshan Hospital of Fudan University, Shanghai, China; [2]Endoscopy Research Institute of Fudan University, Shanghai, China; [3]Department of Internal Medicine of Xuhui Hospital, Affiliated Zhongshan Hospital, Fudan University, Shanghai, China; [4]Endoscopy Center of Xuhui Hospital, Affiliated Zhongshan Hospital, Fudan University, Shanghai, China; [5]General Surgery Department, Zhongshan Hospital, Fudan University, Shanghai, China

Contributions: (I) Conception and design: Q Shi, YS Zhong, JM Xu; (II) Administrative support: Q Shi, YS Zhong, JM Xu; (III) Provision of study materials or patients: YS Zhong, JM Xu; (IV) Collection and assembly of data: ZP Qi, ZH Chen, DL He; (V) Data analysis and interpretation: ZP Qi, ZH Chen, DL He; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Jian-Min Xu, MD, PhD. General Surgery Department, Zhongshan Hospital, Fudan University, Shanghai, China. Email: xu.jianmin1@zs-hospital.sh.cn; Yun-Shi Zhong, MD, PhD; Qiang Shi, MD, PhD. Endoscopy Center, Zhongshan Hospital of Fudan University, Shanghai, 200032, China. Email: zhong.yunshi@zs-hospital.sh.cn; shiqiangqy@126.com.

Background: The CUGBP1 (CELF1) is differentially expressed in liver metastasis and no liver metastasis colorectal cancers (CRC) tissues and the function of CUGBP1 in CRC is still unclear.

Methods: Five cases of colorectal adenocarcinoma and 6 cases of liver metastatic CRC lesions were collected and subjected to cDNA microarray and bioinformatical analyses. The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to confirm the result. Cell function assays were used to study the function of CUGBP1, and the western blot was used to discover the change of the downstream molecules.

Results: CUGBP1 was significantly elevated in liver metastatic CRC lesions. Besides, the CUGBP1 can promote proliferation, colony formation, invasion, metastasis abilities as well as increase the apoptosis rates of CRC cells. ERBB2 was positively related to the CUGBP1. Western blot results found that silence of CUGBP1 decreased the protein level of p-AKT and p-ERK without influence the expression level of total protein of AKT and ERK.

Conclusions: CUGBP1 can promote liver metastasis of CRC by promoting the phosphorylation of AKT and ERK through the ErbB signaling pathway. CUGBP1 is a potential biomarker for early detection of CRC and maybe a novel therapeutic target of CRC treatment, especially in liver metastasis.

Keywords: Colorectal cancer (CRC); liver metastasis; cDNA microarray; Human CUG-binding protein; ErbB signaling pathway

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Introduction

Nowadays, colorectal cancer (CRC) is one of the most frequently diagnosed cancers worldwide (1). CRC arises as a consequence of the accumulation of genetic alterations and epigenetic alterations that transform colonic epithelial cells into adenocarcinoma cells. Liver metastasis is a common

^ ORCID: 0000-0002-3128-3168.

metastasis site of CRC and also the main cause of CRC -related mortality (2). Despite extensive research on the biology of cancer progression, the molecular mechanisms involved in CRC metastasis are not well characterized.

Human CUG-binding protein 1 (CUGBP1), also called CELF1 is a member of the CELF family, it plays a pivotal role in cotranscriptional and posttranscriptional RNA processing (3). In recent years, researchers identified that CUGBP1 is a distinct prognostic value in melanoma (4). The CUGBP1 and DEK were early-induced melanoma genes as well as adverse indicators of poor prognosis (4). CUGBP1 has been frequently reported to promote cell proliferation in several tumors. High expression of CUGBP1 can promote glioma cell proliferation and cell cycle process and it was also found to be a novel independent prognostic predictor of glioma patients (5). However, information regarding the functional role of CUGBP1 in CRC is limited. Besides, there are still some studies that found the CUGBP1 may be a tumor suppressor (6,7). Those phenomena indicated that CUGBP1 may have diverse roles in carcinogenesis, which need better elucidated.

In this study, we screened differentially expressed genes in CRC patients with and without liver metastasis. The RNA binding protein CUGBP1 is the most differentially expressed gene between these two groups. Then we silenced the CUGBP1 in DLD-1 and overexpressed the CUGBP1 in SW480. Results from cell function assays revealed that CUGBP1 can promote cell proliferation, the capability of invasion and metastasis as well as the colony-forming ability and inhibit cell apoptosis. Further, the ERBB2 was positively related to the CUGBP1 and the phosphorylation of AKT and ERK was found to be significantly influenced by silencing CUGBP1. We supposed that the CUGBP1 promotes the progression of CRC by promoting the phosphorylation of AKT and ERK through the ErbB signaling pathway. CUGBP1 may be a potential biomarker for early detection of CRC and a novel therapeutic target of CRC treatment. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/tcr-21-311).

Methods

Cell culture

The human CRC cell lines DLD-1 (TCHu134), SW480 (TCHu172), SW620 (TCHu101), RKO (TCHu116) were obtained from the China Centre for Type Culture Collection, Chinese Academy of Sciences. HCT116 (ATCC® CCL-247) and HT-29 (ATCC® HTB-38) were obtained from the American Type Culture Collection. Cells were cultured following the manufacturer's instructions.

Patient's enrollment

Tumor specimens with clinical and follow-up data were selected from our tumor bank. Patients diagnosed with locally advanced colorectal adenocarcinoma who had no family history of CRC or secondary malignancy and had not received radiation or chemotherapy before surgery were included. Patients in the non-metastatic group had a minimum of 2 years of disease-free survival after surgery. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Y2017-244 and informed consent was taken from all the patients. 200 CRC patients without chemoradiotherapy were continuously enrolled in a year from Zhongshan hospital for survival analysis. All tissue specimens were immediately taken from the operation room upon excision from the patient, snap-frozen in liquid nitrogen, and then stored at –80 ℃ until use.

RNA extraction and microarray scanning and analysis

Five cases of CRC with liver metastases and 6 cases of CRC patients were selected to perform gene chip, the basic information was listed in *Table 1* and *Table 2*. Total RNA was extracted using Trizol reagent (Sigma-Aldrich Inc., Germany). mRNA isolation was then carried out using the QIAquick PCR purification Kit (QIAGEN, United States) according to the manufacturer's instruction. The concentration of the total RNA was measured using the spectrophotometer. Next, the mRNAs from CRC lesions were reverse transcribed into cDNA using Cy5-dUTP labeling, while the mRNAs from the normal mucosae were processed with Cy3-dUTP labeling following the manufacturer's protocols (NEN Company, Boston, MA, United States). The labeled probes were then hybridized to the cDNA microarray (Human Genome U133 Plus 2.0 Array, Affymetrix, Inc., United States). Hybridized cDNA microarrays were scanned using Gene PIX 4000 microarray fluorescence scanner (Axon Instruments, Foster City, CA, United States). Accompanying bioinformatical software was used to convert the output images to data form and perform

Table 1 the general material of the patients doing Gene chip

Stages	Sex	Age (Y)	Site	Maximum diameter (cm)	Pathological type	Differentiation	Stages
A	M	63	Rectal	8	Ulcer type	Medium	T3N1M1 (IV)
C	M	52	Splenic flexure	4	Ulcer type	Medium	T3N1M1 (IV)
D	M	67	Rectal	3	Ulcer type	Medium	T3N2M1 (IV)
F	F	42	Rectal	5	Ulcer type	Medium	T4N1M1 (IV)
E	M	40	Sigmoid colon	4	Ulcer type	Poorly	T4N2M1 (IV)
B	M	54	Sigmoid colon	4	Ulcer type	Medium	T4N0M0 (IIB)
H	M	37	Ascending colon	5	Ulcer type	Medium	T3N0M0 (IIA)
I	M	55	Rectal	3	Ulcer type	Medium	T3N0M0 (IIA)
L	M	64	Rectal	5	Ulcer type	Medium	T4N0M0 (IIB)
K	F	68	Cecum	6	Ulcer type	Medium	T3N0M0 (IIA)
J	M	29	Ascending colon	12	Fungating type	Poorly	T4N0M0 (IIB)

Table 2 Classified data of the patients doing gene chip

	Colorectal cancer with Liver metastasis (sample)	Colorectal cancer without Liver metastasis (control)
Male	A, C, D	B, H, I, L
Female	F	K
Low differentiated adenocarcinoma group	E	J

analysis. Ratios of Cy5:Cy3 was normalized to the median ratio value of all the microarray spots detected. Spots with intensities in both channels that were 0.5 to 2.0-fold higher than the local background were excluded from further analysis. SPSS 13.0 statistical software (Chicago, IL, United States) was used to carry out Student's t-test statistical analysis. P-values <0.05 were considered statistically significant.

Expression and Gene set enrichment analysis (GSEA) analysis

The Oncomine database was used to scanning the general expression of CUGBP1 in CRC. The UALCAN database was used to evaluate the expression level of CUGBP1 in different nodal metastasis statuses. Gene Set Enrichment Analysis (GSEA) was performed using the GSEA software v4.0.3 (https://www.gsea-msigdb.org/gsea/index.jsp). The expression level of CUGBP1 was divided into low and high groups based on the median. KEGG gene sets (c2) and ontology gene sets (c5) from MSigDB were used.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was used to detect the CUGBP1 expression level. CUGBP1 mRNA was amplified by real-time PCR with SYBR green (Invitrogen). PCR was initiated by a 15s denaturation at 95 ℃, followed by 45 cycles of 95 ℃ for 5s, 60 ℃ for 30 s, using an Applied Biosystems 7900HT Sequence Detection System (Foster City, CA, USA). The internal reference of mRNA was GAPDH. The PCR primers used to amplify the CUGBP1 gene were 5'- ACCTGTTCATCTACCACCTG-3' (forward) and 5'- GGCTTGCTGTCATTCTTCG-3' (reverse). GAPDH (a housekeeping gene) primers were 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse).

Protein extraction and Western blotting

Total protein was isolated using a lysis buffer containing PMSF and RIPA. For the staining of CUGBP1, an anti-CUGBP1 mice polyclonal antibody (sc-20003) was

purchased from Santa Cruz Biotechnology, Inc. 30 μg of protein sample were incubated with denaturing buffer (0.3 M Tris pH 6.8, 10% 2-mercaptoethanol, 40% glycerol, 20% SDS, and 0.02% bromophenol blue) at 95 ℃ for 5 min, loaded onto a 10% SDS–polyacrylamide gel for electrophoresis, transferred onto PVDF membranes, and then blocked in 5% non-fat milk/TBS-Tween buffer for 1 h at room temperature. Membranes were then incubated overnight at 4 ℃ in a primary antibody against CUGBP1 (1:500, sc-20003, Santa Cruz), and GAPDH (1:2,000, sc-20357, Santa Cruz). ERBB pathway-related antibodies GSK-3 beta (sc-377213), p- GSK-3 beta (sc-373800), ERK1/2 (sc-514302), p-ERK1/2 (sc-81492), AKT1/2/3 (sc-81434), p-Akt1/2/3 (sc-377556). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoreactivity was detected using chemiluminescence (Pierce, Rockford, IL, USA).

Cell transfection with adenovirus vectors

The siRNA of CUGBP1 was designed and constructed then cloned into the vector pEGFP-N1-3FLAG by repeated excision and ligation successively. The recombinant adenovirus was generated by Genechem CO LTD, Shanghai, China. The particle titers of the adenoviral stocks were 1×10^9 plaque-forming units per milliliter (pfu/mL). Adenovirus vectors expressing CUGBP1 (Ad-CUGBP1). A green fluorescent protein (Ad-GFP) and negative control (Ad-HK) were used to transfect DLD-1 and SW480 cells. Transfection efficiency was evaluated by testing the expression ratio of the green fluorescent protein. The cells were collected after being transfected for 5 d as stably transfected cells.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The proliferation of CRC cells was assessed using the MTT assay. Briefly, transfected DLD-1 or SW480 cells (4×10^3 cells/well) were seeded into 96-well plates and allowed to adhere overnight. At 0 and 24 h, cells in each well were incubated with 20 μL of MTT solution (5 mg/mL) for an additional 2 h at room temperature. The reaction was stopped by adding 100 μL of dimethyl sulfoxide, and the absorbance of each well was measured at 595 nm using a microplate reader (BioTek Instruments, Inc., USA).

Clone formation assay

Cells (200 cells per well) were seeded into six-well plates. The plates were incubated for 2 weeks, and colonies were counted with the assistance of a stereomicroscope.

Flow-cytometry analysis

The apoptosis of CRC cells was determined using flow cytometry. Briefly, transfected DLD-1 or SW480 cells were washed with ice-cold phosphate-buffered saline (PBS) twice and harvested with trypsin. Subsequently, cells were stained with 5 μL of Annexin V-FITC/PI (BC, San Jose, CA, USA) in the dark for 30 min. Finally, the apoptosis rate of stained cells was detected using a BD Accuri TM C6 flow cytometer (BD, Franklin Lakes, NJ, USA).

In vitro matrigel invasion assay

In vitro matrigel invasion assay was performed using a 24-well multicell insert (Corning) with polycarbonate filters (pore size, 8μm). The upper side of the polycarbonate filter was coated with matrigel (50 μg/mL, BD Biosciences). 2×10^5 cells in 200 μL of 0.1% bovine serum albumin serum-free medium were seeded in the upper chamber. The lower chamber was filled with 10% serum-medium (700 μL). Cells were cultured for 24 h at 37 ℃ in 5% CO_2. Cells on the upper surface of the filter were removed using a cotton swab. Chambers were fixed with 4% neutral-buffered formalin for 30 min and stained in Giemsa stain for 15 min. The cell number in five fields (up, down, median, left, and right. ×200) was counted for each chamber, and the average value was calculated. The invasion rate was calculated by the value of MTT read. The GIEMSA staining of the polycarbonate membrane would be digested with acetic acid. Absorbance values (A) were measured at a wavelength of 490 nm with a microplate reader.

Statistical analysis

Statistical analyses were performed using SPSS statistical software (SPSS Inc, Chicago, Illinois). Data were expressed as mean ± SEM. Statistical significance of differences between groups was performed by One Way Analysis of Variance (ANOVA). P-value <0.05 was statistically considered significant. S-N-K was used to analyze the difference between the two groups. All experiments were

conducted at least three times.

Results

Expression and Gene set enrichment analysis (GSEA) analysis

To investigate the role of CUGBP1 in CRC development, we first searched the Oncomine database to examine CUGBP1 expression in CRC tissues. The results found that CUGBP1 was up-regulated in several CRC research (*Figure 1A,B,C*). The UALCAN database showed that the expression levels of CUGBP1 are significantly different among nodal metastasis statuses (*Figure 1D*). Further, GSEA analysis found that the high expression of CUGBP1 is closely related to the regulation of autophagy (NES =2.1550, P<0.0001) and gene silencing (NES =2.5018, P<0.0001) (*Figure 1E,F*).

Validation of CUGBP1 in CRC cells and tissues

We validated the protein expression of CUGBP1 in both tissue and CRC cell lines. As shown in *Figure 2A,B*, CUGBP1 protein was commonly overexpressed (10/12) in CRC tissues and CRC cell lines. IHC results found that the expression of CUGBP1 in the tumor was higher than that in the para tumor (*Figure 2C*). In total, 200 patients diagnosed with CRC and with no liver metastasis were enrolled to evaluate the relationship between the expression of CUGBP1 and the overall survival rate. The low expression of CUGBP1 in the 93 cases, has a median survival time of 20.9 months, and the high expression of CUGBP1 in the 107 cases, has a median survival time of 18.8 months. The low expression of CUGBP1 is beneficial to the overall survival time of CRC patients (P=0.049, χ^2=3.686) (*Figure 2D*).

Clinicopathological assessment of CUGBP1

In CRC tissues, the expression of CUGBP1 was found to be significantly up-regulated in the synchronous live metastasis group, compared to the non-liver-metastasis group (P=0.003). However, there is no difference between metachronous liver metastasis and synchronous live metastasis, and no difference between the synchronous live metastasis and the non-liver-metastasis group. In normal tissue, the expression of CUGBP1 was up-regulated in the metachronous liver metastasis and the synchronous live metastasis obviously, compared to the non-liver-metastasis group (P=0.001, 0.003); there was no obvious difference between the metachronous liver metastasis and the synchronous liver metastasis group. The expression of CUGBP1 was down-regulated (P<0.05), in each group and CRC tissues than the normal tissues (*Table 3*). Wilcoxon rank-sum test was applied to analyze the relationship between the CUGBP1 gene expression and clinical cases characterized. In CRC tissues, there was no significant difference between the expression of CUGBP1 and the clinicopathological correlation (P>0.05). Although in the histological type P values <0.05, considered the imbalance of the number of specimens, the result needed more data to verify (*Table 3*).

We also applied the chi-square test to analyze the relationship between clinicopathological characteristics and different liver metastasis. At the same time, the incidence of liver metastases and metachronous liver metastasis was related to the depth of invasion (P=0.011), vascular invasion (P=0.009), and high CUGBP1 gene expression (P=0.003); the synchronous live metastasis was related to the depth of invasion (P=0.011) and CUGBP1 gene expression (P<0.001) (*Table 4*); the Logistic multivariate regression analysis (Enter method) shows that: vascular invasion were independent risk factors for metachronous liver metastasis. The depth of invasion (T3–T4) and the CUGBP1 gene expression were independent risk factors for synchronous liver metastasis (*Table 5*).

Univariate analysis showed that: colorectal liver metastasis prognosis was related to tumor differentiation, lymph node metastasis, vascular invasion, CUGBP1 expression related (*Table 6*). The univariate analysis, P<0.05 for all the variables included in the multivariate model, derived lymph node metastasis, vascular invasion are independent risk factors for CRC liver metastases prognosis (*Table 7*).

The silence of CUGBP1 influences the cell cycle, proliferation, and invasion abilities of CRC cells

Subsequently, we selected DLD-1 cells (with relatively higher CUGBP1 expression) for transfection with si-CUGBP1 and SW480 cells (with relatively lower CUGBP1 expression) for transfection with oe-CUGBP1, followed by validation of the transfection efficiency. The results of western blot analysis showed that CUGBP1 expression was remarkably decreased after si-CUGBP1 transfection in DLD-1 cells and upregulated after oe-CUGBP1

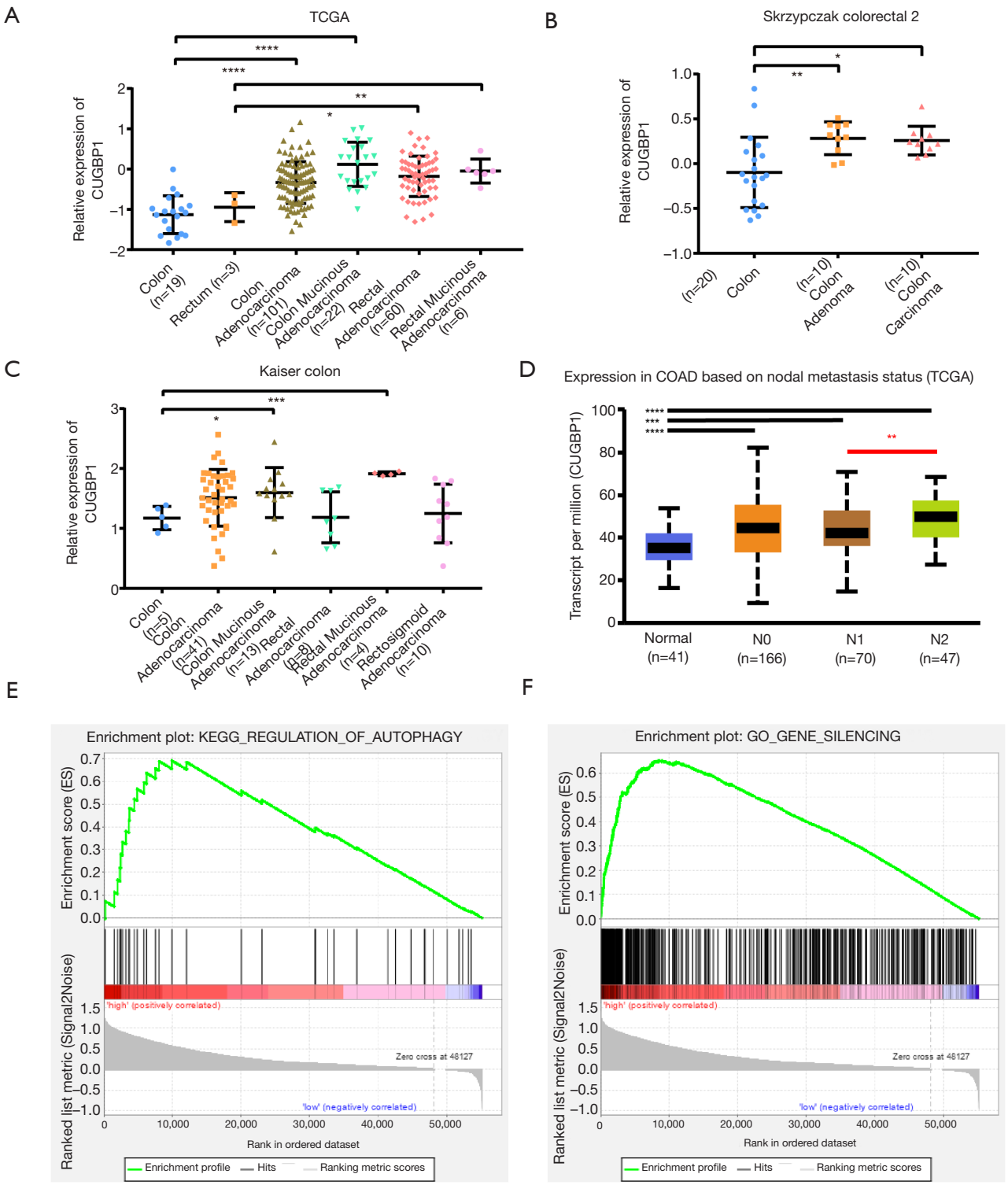

Figure 1 Public databases showed that CUGBP1 is up-regulated in CRC. (A,B,C) Oncomine database showed that CUGBP1 is up-regulated in several colorectal cancer research. (D) different levels of CUGBP1 can help to differentiate the nodal metastasis stage. (E,F) GSEA analysis showed that high expression of CUGBP1 is related to the regulation of autophagy and gene silencing. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

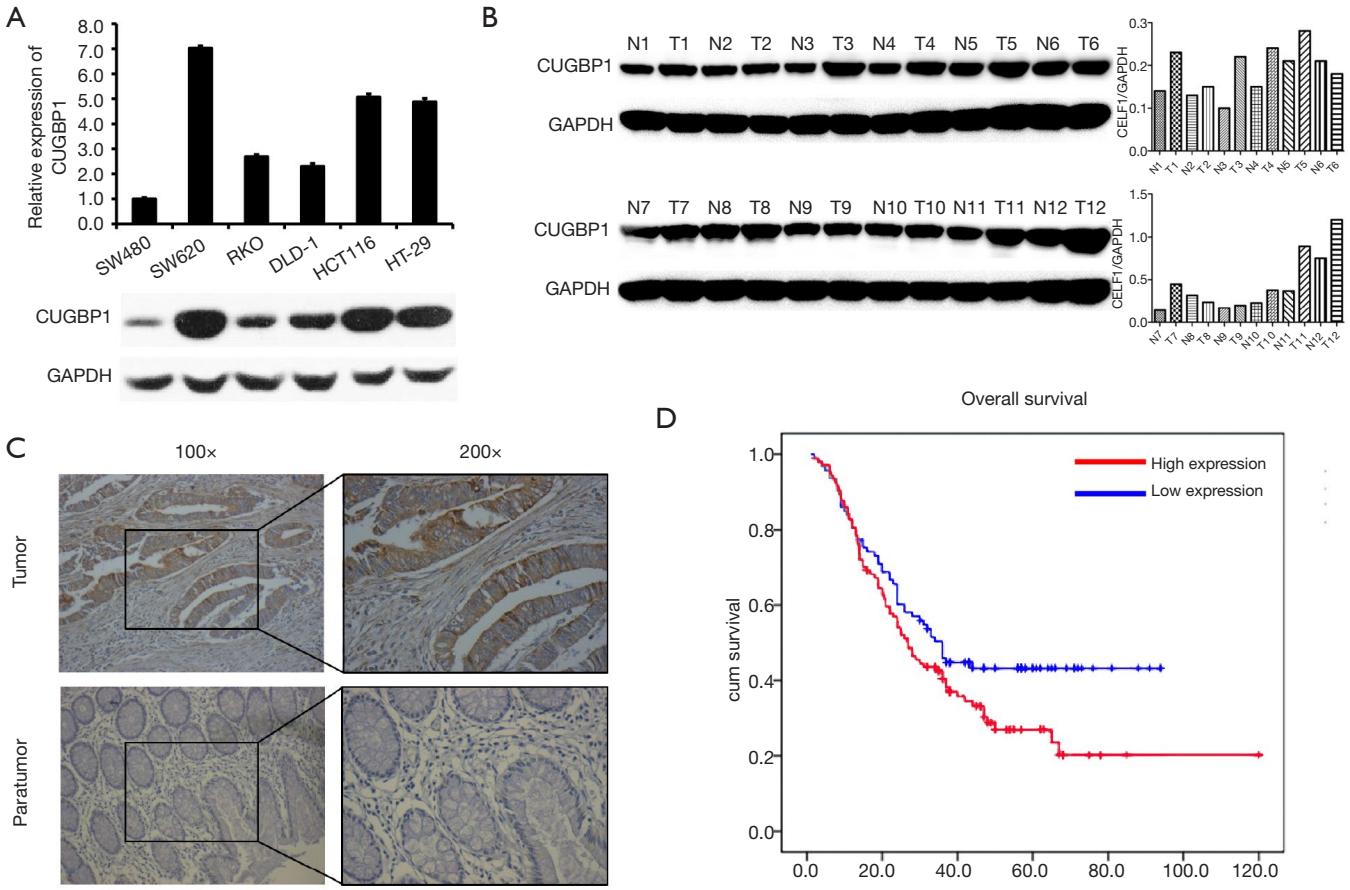

Figure 2 CUGBP1 is up-regulated in CRC cells and tissues. (A) the expression level of CUGBP1 in colorectal cancer cells. (B) the expression level of CUGBP1 in no liver metastasis group, synchronous liver metastasis group, metachronous liver metastasis group, and liver metastasis group. (C) IHC results showed that CUGBP1 is up-regulated in cancer tissue than in para cancer tissue. (D) overall survival analysis found that high expression of CUGBP1 is related to the poor prognosis (P=0.049).

Table 3 The relationship between the expression of CUGBP1 and the clinical pathology

	Score			P	
	Colorectal cancer without liver metastasis	Metachronous liver metastasis	Synchronous live metastasis	Tumor tissue	Normal structure
Colorectal cancer without liver metastasis	20			0.055 [a]	0.001 [a]
Metachronous liver metastasis		20		0.003 [b]	0.003 [b]
Synchronous live metastasis			20	0.636 [c]	0.076 [c]
Sex					
Male	13	9	12	0.441	0.088
Female	7	11	8		

Table 3 (*continued*)

Table 3 (*continued*)

	Score			P	
	Colorectal cancer without liver metastasis	Metachronous liver metastasis	Synchronous live metastasis	Tumor tissue	Normal structure
Age					
<62	12	11	12	0.143	0.532
≥62	8	9	8		
Site					
Colon	15	10	12	0.605	0.212
Rectal	5	10	8		
Size					
<5 cm	13	15	12	0.677	0.823
≥5 cm	4	5	8		
Pathological type					
Fungating type	8	6	7	0.875	0.878
Ulcer type	12	14	13		
Histological type					
Adenocarcinoma	18	19	19	0.044	0.032
Mucinous adenocarcinoma	2	1	1		
Differentiation					
I–II	10	10	9	0.165	0.351
III–IV	10	10	11		
Infiltration depth					
T1–T2	7	5	5	0.534	0.226
T3–T4	13	15	15		
Lymphatic metastasis					
(–)	13	13	15	0.764	0.261
(+)	7	7	5		
Cancerous node					
(–)	14	11	12	0.221	0.547
(+)	6	9	8		
Lymphatic or blood vessel invasion					
No	3	5	3	0.051	0.053
Yes	17	15	17		
Perineural invasion					
No	12	14	11	0.421	0.572
Yes	8	6	9		

[a], colorectal cancer without liver metastasis *vs.* metachronous liver metastasis; [b], colorectal cancer without liver metastasis *vs.* synchronous live metastasis; [c], metachronous liver metastasis *vs.* synchronous live metastasis.

Table 4 the relationship between clinical-pathological characteristics and liver metastasis

Clinical-pathological characteristics	Colorectal cancer without liver metastasis	Metachronous liver metastasis	P (*)	χ^2 (*)	Synchronous live metastasis	P (**)	χ^2 (**)
Sex							
Male	13	9	0.204	1.616	12	0.744	0.107
Female	7	11			8		
Age							
<62	12	11	0.749	0.102	12	0.626	2.440
≥62	8	9			8		
Site							
Colon	15	10	0.102	2.667	12	0.311	1.026
Rectal	5	10			8		
Size							
<5 cm	13	15	0.240	1.380	12	0.744	0.107
≥5 cm	7	5			8		
Pathological type							
Fungating type	8	6	0.507	0.440	7	0.744	0.107
Ulcer type	12	14			13		
Histological type							
Adenocarcinoma	18	19	0.548	0.360	19	0.548	0.360
Mucinous adenocarcinoma	2	1			1		
Differentiation							
I–II	10	10	1.000	0.000	9	0.262	1.245
III–IV	10	10			11		
Infiltration depth							
T1–T2	7	5	0.011	1.317	5	0.011	1.317
T3–T4	13	15			15		
Lymphatic metastasis							
(–)	13	13	0.611	2.180	15	0.240	1.380
(+)	7	7			5		
Cancerous node							
(–)	14	11	0.702	2.134	12	0.294	1.205
(+)	6	9			8		
Lymphatic or blood vessel invasion							
No	3	5	0.009	2.398	3	1.000	0.000
Yes	17	15			17		
Perineural invasion							
No	12	14	0.695	0.333	11	0.221	3.142
Yes	8	6			9		
CUGBP1							
<0.5	19	11	0.003	8.533	7	0.000	15.824
≥0.5	1	9			13		

*, colorectal cancer without liver metastasis *vs.* metachronous liver metastasis; **, colorectal cancer without liver metastasis *vs.* synchronous live metastasis.

Table 5 The factor Influence colorectal cancer hepatic metastasis happens.

	Partial regression coefficient	Standard errors	Wald	P	Relative risks (RRs)	95% confidence intervals (CIs)
Metachronous liver metastasis						
Infiltration depth	–0.307	0.417	1.243	0.301	0.702	1.287–1.652
CUGBP1	–0.524	0.358	1.794	0.137	0.625	0.289–0.985
Lymphatic or blood vessel invasion	–0.709	0.352	6.4070	0.021	0.501	0.231–0.902
Synchronous live metastasis						
Infiltration depth	–0.940	0.401	4.804	0.042	0.399	0.191–0.903
CUGBP1	1.682	0.510	13.998	0.021	4.998	2.301–12.711

Table 6 The single factor analysis of the colorectal liver metastases survival time

	No.	The median survival time	One year survival rate	Three-year survival rate	Five-year survival rate	P
Sex						0.217
Male	21	27	75	33	19	
Female	19	32.2	77	45	16	
Age						0.583
<62	23	29.4	76	40	29	
≥62	17	27.5	75	35	20	
Site						0.061
Colon	22	37.3	83	52	24	
Rectal	18	25.6	74	33	27	
Size						0.835
<5 cm	27	30.4	76	38	22	
≥5 cm	13	24	73	30	14	
Pathological type						0.645
Fungating type	13	30.8	79	36	14	
Ulcer type	27	26.5	75	32	11	
Histological type						0.337
Adenocarcinoma	38	29.8	75	44	21	
Mucinous adenocarcinoma	2	27.8	72	33	17	
Differentiation						0.046
I–II	19	39.5	77	42	23	
III–IV	21	26.2	75	34	19	

Table 6 (*continued*)

Table 6 (*continued*)

	No.	The median survival time	One year survival rate	Three-year survival rate	Five-year survival rate	P
Infiltration depth						0.345
T1–T2	10	27.1	74	32	20	
T3–T4	30	29.9	77	42	25	
Lymphatic metastasis						0.028
(–)	28	39.6	76	43	17	
(+)	12	27.1	71	33	10	
Cancerous node						0.69
(–)	23	29.7	76	40	19	
(+)	17	28.2	73	37	12	
Lymphatic or blood vessel invasion						0.001
No	8	23.1	98	38	17	
Yes	32	19.3	69	21	5	
Perineural invasion						0.365
No	25	31.4	78	42	27	
Yes	15	24.7	73	33	15	
CUGBP1						0.012
<0.5	18	22.5	78	56	41	
≥0.5	22	18.2	71	39	27	

Univariate analysis was calculated using the Kaplan–Meier method (the log-rank test). HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis.

Table 7 Multivariate analysis of the colorectal liver metastases prognosis.

Survival time	P	HR (95% CI)
Differentiation (I/II *vs.* III/IV)	NS	
Lymphatic metastasis (no *vs.* yes)	0.021	1.921 (1.354–2.873)
Lymphatic or blood vessel invasion (no *vs.* yes)	0.018	2.282 (1.099–4.452)
CUGBP1 low expression *vs.* CUGBP1 high expression	0.058	1.672 (1.089–2.385)

Multivariate analysis was performed using the Cox multivariate proportional hazard regression model with a stepwise method (backward, likelihood ratio).

transfection in SW480 cells (*Figure 3A*,*B*). In the flow cytometry experiments, the percentages of cells in early and late apoptosis were significantly elevated in DLD-1 cells after CUGBP1 knockdown. In contrast, CUGBP1 overexpression notably decreased the apoptosis rate in SW480 cells (*Figure 3C*). Colony assays showed that the silence of CUGBP1 can weaken the clone formation ability of DLD-1 and SW480 (*Figure 3D*,*E*). The MTT assay found that the silence of CUGBP1 can significantly reduce the proliferation ability of DLD-1 and SW480 (*Figure 3F*). And transwell assays found that the silence of CUGBP1 can weaken the invasion ability of both DLD-1 and SW480. These findings suggest that the expression level of CUGBP1 seems to be closely associated with the enhancement of the progression and invasion of DLD-1and SW480 (*Figure 3G*).

CUGBP1 may influence the cell cycle, apoptosis, and invasion of CRC cells through the ErbB signaling pathway

Further, based on the COAD dataset from the TCGA database, the expression of CUGBP1 was found positively related to ERBB2 with R=0.35 (P<0.001) (*Figure 4A*). The expression of ERBB2 was evaluated in the CUGBP1 silenced DLD-1 cell, the result also confirmed that both CUGBP1 and ERBB2 were decreased in DLD-1 (*Figure 4B*). Western blot experiments showed that the silencing of CUGBP1 could significantly inhibit the expression of the phosphorylation of AKT and ERK, which are well-known downstream targets of the ErbB signaling pathway. Meanwhile, the total protein expression levels of AKT and ERK did not change obviously in transfected DLD-1 cells. (*Figure 4C*,*D*). The regulation mechanism graph was constructed in *Figure 4E*.

Conclusions

CRC is the world's fourth most deadly cancer and caused considerable morbidity and mortality (8). Though numerable treatment options were available, the prognosis of metastasis CRC patients is significantly worse than that of non-metastasis CRC patients (8). Nowadays, much is known about the genes that are affected by cancer-causing mutations in CRC and those clinically relevant biomarkers help to manage the CRC (9), however, less is known about molecular events that are crucial for metastasis formation. The liver is the most common metastasis site of CRC (2). In this study, we explored the mechanism of liver metastasis of CRC and found that the CUGBP1 plays an important role during the process of liver metastasis.

Primarily, the microarray found a set of 110 genes that can separate metastatic primary tumors from nonmetastatic primary tumors. Of these genes, 60 were up-regulated (55 was known), and 50 were down-regulated (43 was known). Among the differentially expressed genes, CUGBP1 was the only one that was up-regulated in most CRC researches and expressed significantly different among lymph node metastasis statuses. Besides, GSEA analysis found that high expression of CUGBP1 is related to the regulation of autophagy, which is closely related to liver metastasis (10). Human CUG-binding protein 1 (CUGBP1) is one of the key members of the CELF/BRUNOL protein family, whose function in both regulate pre-mRNA alternative splicing and in mRNA editing as well as translation (11,12). Recently, researchers found that CUGBP1 can enhance CRC cells' migration, invasion, and chemoresistance ability by targeting ETS2 (13).

CUGBP1 expression in CRC tissues is higher than in the para cancer tissues. It may relate to the occurrence of CRC and heterochrony liver metastasis. Colorectal carcinoids with low expression of CUGBP1 exhibited no LN metastasis or distant metastasis. Overall survival of patients with high expression of CUGBP1 was poor than that with low expression. However, the survival was similar between carcinoids and adenocarcinomas if the tumors had LN metastasis or distant metastasis. This study also hints that the high expression of CUGBP1 is the independent risk factor of the CRC heterochrony liver metastasis. In terms of cell function assays, the silencing of CUGBP1 can inhibit the proliferation and colony-forming ability of SW480 and DLD-1 cells, and the apoptosis was increased. At the same time, the capability of invasion and metastasis was significantly inhibited, which indicates that the CUGBP1 mediates liver metastasis by promoting the proliferation, invasion, and metastasis ability of CRC. This finding was consistent with the previous researches (5,14). In-depth, we study the downstream regulation mechanism of CUGBP1. Interestingly, the expression of ERBB2 is positively related to CUGBP1. ERBB2 is a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases that are overexpressed in most cancers. It has already been the target of several cancer therapies (15-17). Western blot results showed silence of CUGBP1 can decrease the expression of p-AKT and p-ERK. AKT is a serine/threonine kinase that plays a pivotal role in the PI3K signaling pathway, and it is frequently deregulated in

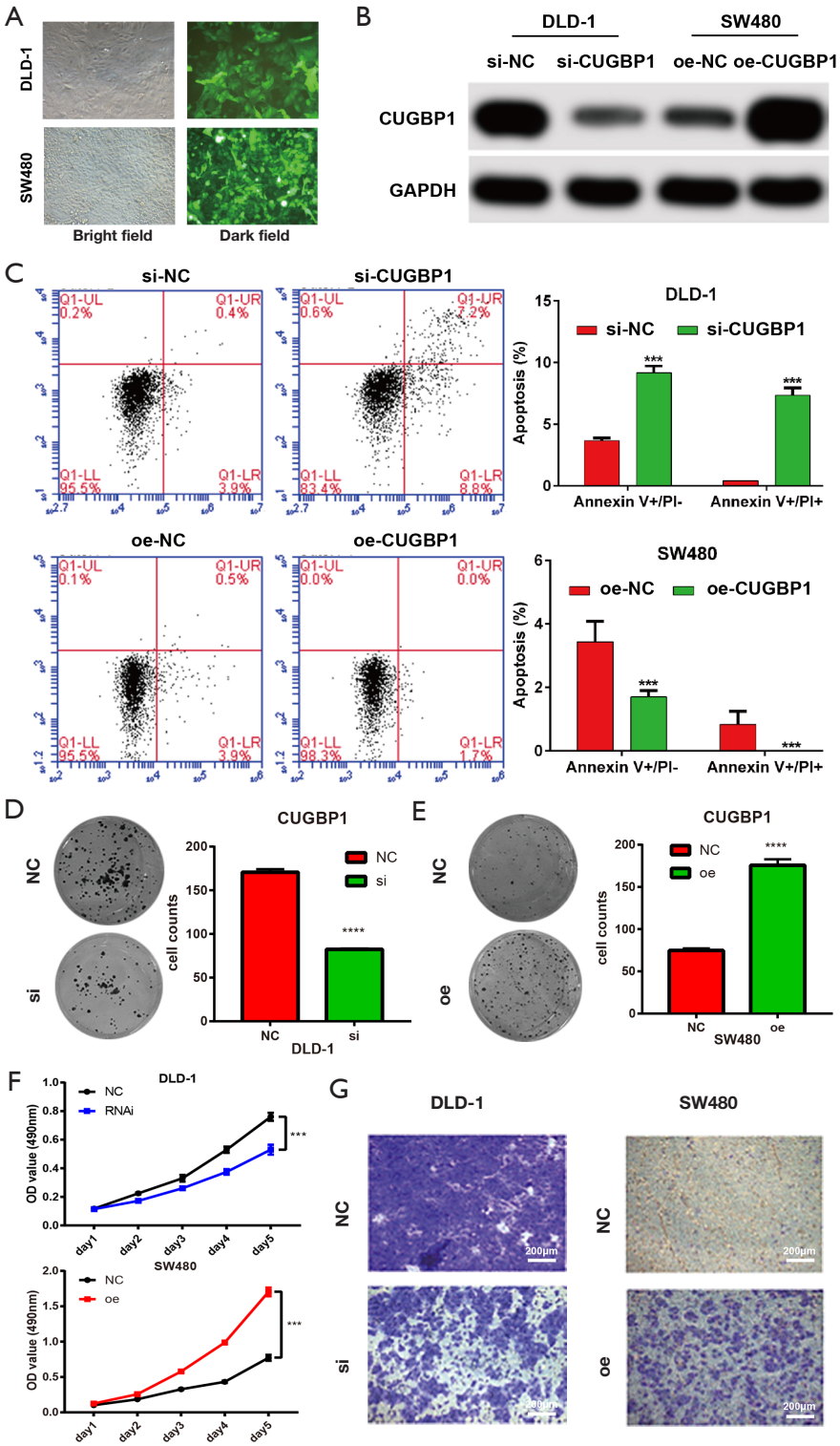

Figure 3 The silencing of CUGBP1 can inhibit the cell cycle, proliferation, and invasion of CRC cells. (A,B) The CUGBP1 was successfully silenced and overexpressed in DLD-1 and SW480. (C) CUGBP1 can inhibit apoptosis. (D,E) CUGBP1 can promote clone formation ability. (F) CUGBP1 can improve the proliferation ability. (G) Giemsa staining showed that CUGBP1 can promote invasion ability (200 μm). ***, P<0.001; ****, P<0.0001.

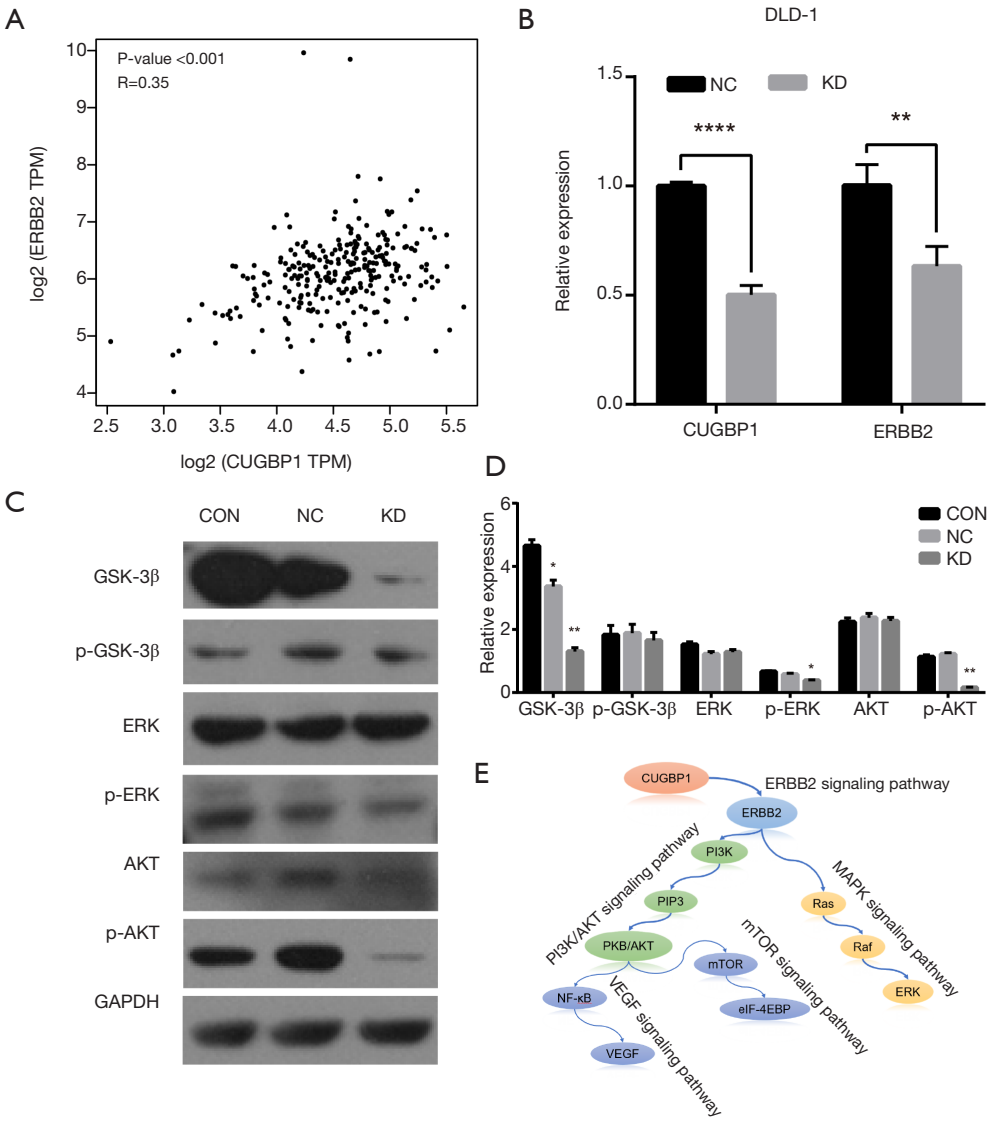

Figure 4 CUGBP1 may influence the progress of CRC through the ErbB signal pathway. (A) The expression of CUGBP1 is positively related to the ERBB2 (based on TCGA cohort). (B) silencing of CUGBP1 resulted in the decrease of ERBB2. (C) silencing of CUGBP1 decreased the expression of the phosphorylation of AKT and ERK without changing their total protein expression. (D) Gray value analysis of the Western blot bands. E: CUGBP1 may mediate the liver metastasis of colorectal cancer by regulating the PI3K/AKT and MAPK signaling pathway through the ErbB signaling pathway. *, P<0.05; **, P<0.01; ****, P<0.0001.

human cancers (18). Studies found that ERBB2 can activate both the Ras/MAPK and PI3K/AKT signal pathway, which plays an important role in the progression and prognosis of cancers (19,20), especially in cell invasion and migration (21,22). Thus, CUGBP1 may promote liver metastasis of CRC by activating MAPK and PI3K pathways through the ErbB2 receptor. ERK1/2 is a member of the MAP kinase family that plays an important role in tumor proliferation, invasion, and metastasis (23). Elevated levels of p-AKT (24-26) and p-ERK (27) have been frequently detected in cancers. In a word, based on the positive effects of p-AKT and p-ERK on the proliferation of CRC cells, we supposed that CUGBP1 can promote CRC growth by upregulating the ErbB signal pathway.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Y2017-244 and informed consent was taken from all the patients.

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