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
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 °C 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
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 °C, followed by 45 cycles of 95 °C for 5s, 60 °C 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 used.

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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                                                   |

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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 °C 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 °C 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 (4×10^4 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 °C 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 G1EMS 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, $\chi^2$=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 liver 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               | Tumor tissue | Normal structure |
|----------------------------|-----------------|--------------|-----------------|
| Colorectal cancer without liver metastasis | 20              | 0.055<sup>a</sup> | 0.001<sup>a</sup> |
| Metachronous liver metastasis | 20              | 0.003<sup>b</sup> | 0.003<sup>b</sup> |
| Synchronous live metastasis | 20              | 0.636<sup>c</sup> | 0.076<sup>c</sup> |
| Sex                        |                 |              |                 |
| Male                       | 13              | 0.441        | 0.088           |
| Female                     | 7               | 11           | 8               |

Table 3 (continued)
Table 3 (continued)

|                      | Score |                      |                      | P          |                      | Tumor tissue | Normal structure |
|----------------------|-------|-----------------------|----------------------|------------|----------------------|--------------|------------------|
|                      |       | Colorectal cancer     | Metachronous liver   |            | Synchronous live     |              |                  |
|                      |       | without liver         | metastasis          |            | metastasis          |              |                  |
|                      |       | 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 | 3 | 5 | 3 | 0.051 | 0.053 |
| No                   | 17    | 15                    | 17                   |            |                      |              |                  |
| Yes                  | 3     | 5                     | 3                    | 0.051      | 0.053                |              |                  |
| Perineural invasion  |       |                       |                      |            |                      |              |                  |
| No                   | 12    | 14                    | 11                   | 0.421      | 0.572                |              |                  |
| Yes                  | 8     | 6                     | 9                    |            |                      |              |                  |

*coloctral cancer without liver metastasis vs. metachronous liver metastasis; †, colorectal cancer without liver metastasis vs. synchronous live metastasis; ‡, 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 (*) | $\chi^2$ (*) | Synchronous liver metastasis | P (***) | $\chi^2$ (***) |
|--------------------------------------|------------------------------------------|-------------------------------|-------|-------------|-------------------------------|---------|--------------|
| 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 liver 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  | 6.407| 0.021                | 0.501                         | 0.231–0.902                   |
| Synchronous liver 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 |                          |                        |                          |                         |    |
| Male | 21                       | 27                     | 75                       | 33                      | 19 |
| Female | 19                      | 32.2                   | 77                       | 45                      | 16 |
| Age |                          |                        |                          |                         |    |
| <62 | 23                       | 29.4                   | 76                       | 40                      | 29 |
| ≥62 | 17                       | 27.5                   | 75                       | 35                      | 20 |
| Site |                          |                        |                          |                         |    |
| Colon | 22                       | 37.3                   | 83                       | 52                      | 24 |
| Rectal | 18                      | 25.6                   | 74                       | 33                      | 27 |
| Size |                          |                        |                          |                         |    |
| <5 cm | 27                       | 30.4                   | 76                       | 38                      | 22 |
| ≥5 cm | 13                       | 24                     | 73                       | 30                      | 14 |
| Pathological type |            |                        |                          |                         |    |
| Fungating type | 13               | 30.8                   | 79                       | 36                      | 14 |
| Ulcer type | 27             | 26.5                   | 75                       | 32                      | 11 |
| Histological type |            |                        |                          |                         |    |
| Adenocarcinoma | 38            | 29.8                   | 75                       | 44                      | 21 |
| Mucinous adenocarcinoma | 2        | 27.8                   | 72                       | 33                      | 17 |
| Differentiation |            |                        |                          |                         |    |
| 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** |     |                          |                        |                          |                         |    |
| T1–T2             | 10  | 27.1                     | 74                     | 32                       | 20                      |    |
| T3–T4             | 30  | 29.9                     | 77                     | 42                       | 25                      |    |
| **Lymphatic metastasis** |     |                          |                        |                          |                         |    |
| (-)               | 28  | 39.6                     | 76                     | 43                       | 17                      |    |
| (+)               | 12  | 27.1                     | 71                     | 33                       | 10                      |    |
| **Cancerous node** |     |                          |                        |                          |                         |    |
| (-)               | 23  | 29.7                     | 76                     | 40                       | 19                      |    |
| (+)               | 17  | 28.2                     | 73                     | 37                       | 12                      |    |
| **Lymphatic or blood vessel invasion** |     |                          |                        |                          |                         |    |
| No                | 8   | 23.1                     | 98                     | 38                       | 17                      |    |
| Yes               | 32  | 19.3                     | 69                     | 21                       | 5                       |    |
| **Perineural invasion** |     |                          |                        |                          |                         |    |
| No                | 25  | 31.4                     | 78                     | 42                       | 27                      |    |
| Yes               | 15  | 24.7                     | 73                     | 33                       | 15                      |    |
| **CUGBP1**        |     |                          |                        |                          |                         |    |
| <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-1 and 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.
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.

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.
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