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Original Article

Zinc finger and BTB domain-containing 7C (ZBTB7C) expression as an independent prognostic factor for colorectal cancer and its relevant molecular mechanisms

Xuenuo Chen[1], Zhongxiang Jiang[2], Yihuan Pu[2], Xiaoye Jiang[1], Lichun Xiang[1], Zheng Jiang[1]

[1]*Department of Gastroenterology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, P. R. China;* [2]*Chongqing Medical University, Chongqing 400016, P. R. China*

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Abstract: Currently, colorectal cancer (CRC) predictions are based on an early diagnosis and the tumor-node-metastasis (TNM) stage, but the outcomes of patients with the same cancer type are difficult to predict. Novel molecular tests for the early diagnosis and stratification of CRC patients must be devised. After our initial bioinformatics screen, we examined zinc finger and BTB domain-containing 7C (ZBTB7C). To date, few studies have investigated ZBTB7C in CRC, necessitating further analyses of its expression and regulatory mechanism in CRC. ZBTB7C mRNA and protein expression was detected in CRC and corresponding non-CRC tissues. We evaluated the relationship between clinical prognosis and ZBTB7C protein levels using Cox regression analysis and Kaplan-Meier curves. A receiver operating characteristic (ROC) curve was generated to verify the diagnostic performance of ZBTB7C levels in CRC. Several bioinformatics techniques were applied to analyze the potential molecular mechanism of ZBTB7C. Low mRNA and protein levels of ZBTB7C were detected in tumor tissues from CRC patients. The survival curve predicted a poor prognosis for CRC patients exhibiting low ZBTB7C expression (P=0.001). According to the univariate Cox regression analysis, older age, a high TNM stage and low ZBTB7C expression were responsible for poor outcomes in CRC patients. The multivariate analysis further revealed ZBTB7C as an independent prognostic factor for CRC (P=0.015). The area under the curve of ZBTB7C expression for CRC diagnosis was 0.970 (95% confidence interval, 0.9447-0.9946; P < 0.0001). According to in silico analyses, genes coexpressed with ZBTB7C are associated mainly with the Ras and Wnt signaling pathways. Overall, ZBTB7C is downregulated in CRC and represents an early diagnostic marker and independent prognostic factor for CRC. ZBTB7C may be functionally mediated by different pathways or targeting miRNAs.

Keywords: ZBTB7C, colorectal cancer, survival, independent prognostic factor, biomarker

Introduction

Colorectal cancer (CRC) is the third most common malignant tumor in the world and the second leading cause of cancer-related death. Notably, 1.8 million new cases and 900,000 deaths occur each year [1]. In particular, in 2018, 10 of every 10,000 people in Asia were affected by CRC, and the incidence (51.8%) and mortality (52.4%) of CRC ranked first in the world. In addition, the incidence of the disease is increasing throughout Asia [2]. Diagnosis and treatment are two major obstacles to overcome CRC. On the one hand, the early symptoms of CRC are not obvious, the diagnosis is often missed, metastasis often occurs, treatment is difficult, and the prognosis is poor. After radical resection of early CRC, the 5-year survival rate of patients reaches more than 90%, while the 5-year survival rate of patients with advanced cancer is less than 10%. On the other hand, tumor-node-metastasis (TNM) stage, the most widely used prognostic predictor, is unable to keep pace with the current complex clinical oncology environment. For example, when considering whether to use adjuvant therapy in patients with stage II CRC, clinicians must consider whether the toxicity is greater than the

benefit. Therefore, a new independent prognostic factor that diagnoses CRC early and guides clinical management is urgently needed.

The zinc finger and BTB domain-containing protein (ZBTB) family is a class of proteins that contain a BTB domain at the N-terminus and numerous zinc finger domains at the C-terminus [3]. The human genome encodes at least 49 of these proteins, most of which are nuclear proteins. The BTB domain mediates the formation of homologous/heteromultimers or interactions with other proteins [1]. The C-terminal zinc finger structure is generally responsible for binding to specific DNA sequences, and therefore, most ZBTB family members function as transcription factors [4]. Members of the ZBTB family play a key role in the development of multiple tumors. For example, BCL6 (ZBTB27) has been shown to be a proto-oncogene in glioblastoma and diffuse large B-cell lymphoma [5, 6]. Furthermore, ZBTB7 may be important in the development and progression of transitional cell carcinoma (TCC) [7], and Kaiso (ZBTB33) alters MTG16-driven inflammation and tumorigenesis [8].

ZBTB7C is a candidate tumor suppressor gene discovered by Stella Reuter in 1998. The study indicated that this gene may be expressed at low levels or not expressed in most cervical cancer cell lines, but it is expressed in normal cervical epithelial cells. In addition, colony formation experiments verified its ability to inhibit growth [9]. However, several studies have shown that ZBTB7C functions as a proto-oncogene by supporting the rapid proliferation of human renal clear cell carcinoma cells, increasing glutamine uptake and fatty acid synthesis and inhibiting p53 [10-13]. As a key transcription factor, ZBTB7C is involved in various metabolic pathways. For example, it inhibits the expression of the MMP gene and regulates fasting blood glucose levels [14, 15]. However, we currently know very little about ZBTB7C, which plays very different roles in different tumor cells and requires further study. The clinical significance and mechanism regulating its expression in CRC are unclear. Therefore, further research is needed to investigate the expression of ZBTB7C in CRC and its possible regulatory mechanisms to provide new insights into the diagnosis and treatment of CRC.

In our study, the levels of the ZBTB7C mRNA and protein were detected in CRC and paracancerous tissues using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC). The expression of ZBTB7C in CRC was verified using the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. At the same time, using a variety of bioinformatics and statistical methods, we performed a comprehensive analysis of the role of ZBTB7C in CRC, including early diagnosis, prognostic relationship, possible molecular mechanisms, hub genes, and potential target microRNAs. The study design is shown in **Figure 1**.

Materials and methods

Patients

Twenty-seven fresh CRC and adjacent tissues excised from patients were collected from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) from January 2019 to June 2019 for RT-qPCR. Furthermore, 69 pairs of paraffin sections preserved in the Pathology Department of Chongqing Medical University from June 2018 to March 2019 were collected for immunohistochemistry. None of the patients received radiotherapy before surgery. All samples were collected with the informed consent from the patients and their families, and the procedures were reviewed by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

RT-qPCR

Total RNA was extracted from 27 pairs of fresh colorectal cancer and paracancerous tissues using TRIzol Reagent (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The total RNA was reversed transcribed to cDNAs using a PrimeScript™RT reagent kit (TaKaRa Biotechnology Co., Ltd.). PCR was then performed according to the instructions provided with the qPCR reagent. The primers were designed by TaKaRa Biotechnology Co., Ltd., and the following sequences were used: ZBTB7C: forward, 5'-CCACGAAACTACCTTCAACTCC-3' and reverse, 5'-GTGATCTCCTTCTGCATCCTGT-3' (155 bp); and β-actin: forward, 5'-CCACGAAACTACCTTCAACTCC-3' and reverse, 5'-GTGATCTCCTTCTGCATCCTGT-3' (132 bp). Expression was normalized to β-actin,

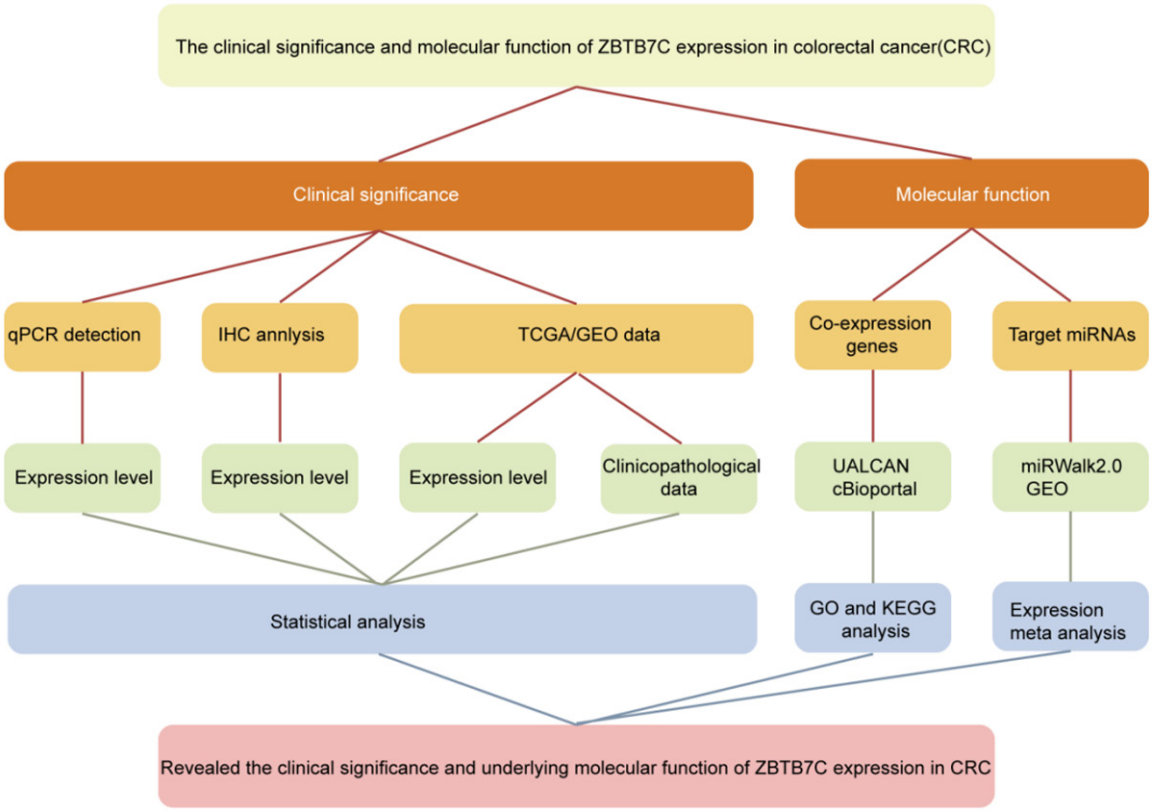


Figure 1. Study design and flow diagram. First, we used RT-qPCR and IHC to reveal the expression level of ZBTB7C in CRC. Then, the expression of ZBTB7C was verified by TCGA and geo microarray sequencing data. Then, the relationship between the expression of ZBTB7C and the diagnosis and survival grade was evaluated by using the clinical data related to the chip. Finally, the potential molecular mechanism of ZBTB7C was analyzed by using a variety of bioinformatics methods.

where the blank control was set to 0, and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [16].

Immunohistochemistry (IHC)

Paraffin sections of CRC tissue samples (n=69) were incubated at 60°C for 120 minutes, followed by sequential incubations in xylene I for 15 min, xylene II for 15 min, xylene III for 15 min, absolute ethanol I for 5 min, absolute ethanol II for 5 min, 85% alcohol for 5 min, 75% alcohol for 5 min, and distilled water. Then, the tissue sections were placed in a repair kit filled with EDTA (pH 9.0) antigen repair solution (G1203; Wuhan Servicebio Saiwei Biotechnology Co., Ltd.) for antigen retrieval in a microwave oven, heated at medium power for 10 min to boil the samples, cooled for 10 min, heated a medium and low power for 5 min, cooled for 2 min, and then heated at low power for 5 min. After natural cooling, the slides were placed in PBS (pH 7.4) and washed three times for 5 min each on a decolorizing shaker. Sections were placed in a 3% aqueous solution of hydrogen peroxide to block endogenous peroxidases, incubated at room temperature for 25 min in the dark, and washed with PBS (pH 7.4) 3 times for 5 min each on a bleaching shaker. Next, 3% BSA was added dropwise to the tissue circle to uniformly cover the tissue, and the sections were blocked at room temperature for 30 min. The blocking solution was gently removed, the rabbit monoclonal antibody (Bs-13583R; anti-ZBTB7C antibody; 1:200 dilution; Beijing Biosynthesis Biotechnology Co., Ltd., China) was added to the section, and incubated in a humid chamber at 4°C overnight. Subsequently, the slides were washed with PBS (pH 7.4) three times for 5 min each on a decolorizing shaker. After the sections were slightly dried, the tissues were covered with HRP-labeled rabbit anti-goat antibody (GB23204; 1:200 dilution; Wuhan Servicebio Biotechnology Co., Ltd.), and

incubated for 50 min at room temperature. A freshly prepared DAB coloring solution was added dropwise in the circle, and the color development time was controlled under a microscope. A positive color was brownish yellow, and the sections were washed with tap water to terminate color development. Hematoxylin counterstaining was performed for approximately 3 minutes, sections were washed with tap water, and the hematoxylin differentiation solution was incubated with the sections for several seconds. The sections were rinsed with tap water, and the hematoxylin blue liquid returned to blue. Finally, the sections were sequentially incubated in 75% alcohol for 5 min, 85% alcohol for 5 min, absolute ethanol I for 5 min, absolute ethanol II for 5 min, and xylene I for 5 min to dehydrate the sections and render them transparent. The slices were removed from the xylene and dried slightly, followed by sealing with neutral gum. Images were captured using a Leica microscope imaging system (100× and 400× magnification; Leica Microsystems GmbH, Wetzlar, Germany). The data were assessed by two independent pathologists who were blinded to the samples. A semiquantitative immunoreactivity scoring system was used to sort patients into high and low expression groups according to their immunoreactivity score [17, 18].

The GEO and TCGA databases

The public gene expression profile GSE32323 was downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo), a public functional genomic data repository. GSE32323 contains 17 pairs of cancer and noncancerous tissues from patients with CRC, as measured using Affymetrix HG-U133 Plus 2.0 arrays. The data on ZBTB7C expression (level 3 data, log2 transformed) from the TCGA database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) were downloaded with the Genomic Data Commons (GDC) Data Portal (https://portal.gdc.cancer.gov/), which provided a normalized count of the gene-level transcription in 425 CRC and 36 noncancerous tissues. The clinicopathological data, including the age at the initial diagnosis, sex, T stage, M stage, N stage, survival status and overall survival time, were downloaded for reanalysis. All patients (n=425) were divided into low and high expression groups according to the median value of ZBTB7C expression. All data were preprocessed with R 3.6.0.

Multiplatform analysis

The Oncomine online tool (www.oncomine.org/, based on the TCGA database) was used to identify and confirm the correlation of ZBTB7C expression with cancers or CRC [19]. cBioPortal (online tool, www.cbioportal.org, based on the TCGA database) was used to identify the mutation status of the ZBTB7C gene [20]. The OncoLnc (http://www.oncolnc.org, based on the TCGA database) and Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/, a visualization website based on TCGA data) online tools were used to analyze the survival of patients with CRC presenting different levels of ZBTB7C expression. miRWalk2.0 (http://zmf.umm.uni-heidelberg.De/apps/zmf/mirwalk2/) was used to predict the target miRNAs and contains 12 online prediction tools; more than 7 miRNAs were predicted targets.

Gene set enrichment analysis (GSEA)

The analysis was performed using GSEA 3.0 [21]. ZBTB7C expression was divided into high expression and low expression. Using c2.cp.kegg.v6.2.symbols.gmt in the GSEA database as the functional gene set, the default weighted enrichment statistics method was used to set the random combination number to 1000, and the association of the ZBTB7C expression level with various biological pathways was analyzed. The impact of the gene set was determined by the following cut-off criteria: $P < 0.05$, gene size ≥ 15, false discovery rate (FDR) < 0.25, and $|\text{enrichment score (ES)}| > 0.5$.

Genes coexpressed with ZBTB7C

We included genes coexpressed with ZBTB7C in this study to further explore and refine the potential mechanism of ZBTB7C in CRC. The genes coexpressed with ZBTB7C in CRC were screened based on UALCAN (http://ualcan.path.uab.edu/) and the cBioPortal database with an absolute value of Pearson's correlation coefficient ≥ 0.3 as the cut-off value. Next, CRC-related genes were screened in GeneCards (https://www.genecards.org/). Finally, the intersecting results among UALCAN, cBioPortal and GeneCards were obtained by FunRich 3.1.3 (Figure S1).

Bioinformatics analyses

We performed a series of bioinformatics analyses on the previously mentioned genes coex-

pressed ZBTB7C. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using clusterProfiler R package and KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/). The protein-protein interaction (PPI) analysis was performed using the search tool for the retrieval of interacting genes/proteins (STRING) v11.0 (https://string-db.org/) database. Hub genes were identified using Cytoscape 3.7.1 software and the cytoHubba plugin.

Statistical analysis

Statistical analyses were performed using R 3.6.0 and SPSS 25.0. Plots of the statistical results were generated using GraphPad Prism 8.0.2 and R 3.6.0. All experiments were performed in triplicate. A chi-square test was used to analyze the immunohistochemistry results and to assess the correlation between ZBTB7C expression and the clinicopathological parameters of CRC. A Kaplan-Meier analysis was performed to study the association between ZBTB7C expression levels and patient survival. Univariate and multivariate Cox regression analyses were conducted to assess the prognosis of patients with CRC presenting different levels of ZBTB7C expression. We calculated the area under the receiver operating characteristic (ROC) curve (AUC) to evaluate the diagnostic value of ZBTB7C expression in CRC. A two-tailed $P < 0.05$ indicated that the difference was statistically significant.

Results

Expression of ZBTB7C in CRC tissues

Levels of the ZBTB7C mRNA and protein were detected using RT-qPCR and IHC, respectively. The RT-qPCR results showed significantly lower expression of the ZBTB7C mRNA in 27 CRC tissues than in the normal control tissues ($P < 0.05$; **Figure 2A**). Similar changes protein levels were detected to the mRNA expression, and the levels of the ZBTB7C protein were significantly decreased in CRC tissues compared to the matched paracancerous tissues (n=69; $P < 0.0001$; **Figure 2D** and **Table 1**).

ZBTB7C expression data obtained from TCGA and GEO databases were analyzed to validate these results. The changes in ZBTB7C expression were consistent with the RT-qPCR and IHC results ($P < 0.0001$, **Figure 2B**; and $P < 0.0001$, **Figure 2C**). Simultaneously, this differential expression pattern was also confirmed in 19 datasets in the Oncomine database (**Figure 2E**).

Furthermore, mining the cBioPortal data revealed alterations in ZBTB7C expression in 33/594 (6%) sequenced samples, with 1 case of amplification, 8 cases of missense mutations, 6 cases of deep deletions, 11 cases of mRNA upregulation, 1 case of an in frame mutation and 6 cases of truncating mutations (Figure S2). A summary and analysis of the data revealed that the somatic mutation frequency of ZBTB7C was 2.9%, and missense mutations were the most common type of mutation in CRC (Figure S3).

Low ZBTB7C expression as a potential diagnostic biomarker of CRC

Based on TCGA data, we investigated the feasibility of ZBTB7C as a diagnostic indicator of CRC. ZBTB7C expression was strongly correlated with CRC ($r=-0.436$; $P < 0.0001$). At the same time, the ROC curve confirmed a high diagnostic value of ZBTB7C expression for the differential diagnosis of CRC and normal tissues. The AUC was 0.970 (95% CI, 0.9447-0.9946; $P < 0.0001$), the sensitivity was 95.5%, the specificity was 88.9%, and the diagnostic threshold was 7.393 (**Figure 2F**).

Correlation of ZBTB7C expression with clinicopathological parameters of patients with CRC

Next, we investigated the relationship between ZBTB7C expression levels and the clinicopathological parameters of 425 patients with CRC. As shown in **Table 2**, the downregulated expression of ZBTB7C in CRC tissues was unrelated to the clinicopathological parameters of patients with CRC (**Table 2**).

Relationship between ZBTB7C expression and the prognosis of patients with CRC

A Kaplan-Meier survival curve was plotted for the 425 patients with CRC to evaluate the relationship between ZBTB7C expression and the prognosis of patients with CRC. As shown in **Figure 3A**, the overall survival was significantly different between ZBTB7C-positive and ZBTB7C-negative patients ($P=0.001$), suggesting that low ZBTB7C expression predicts a poor

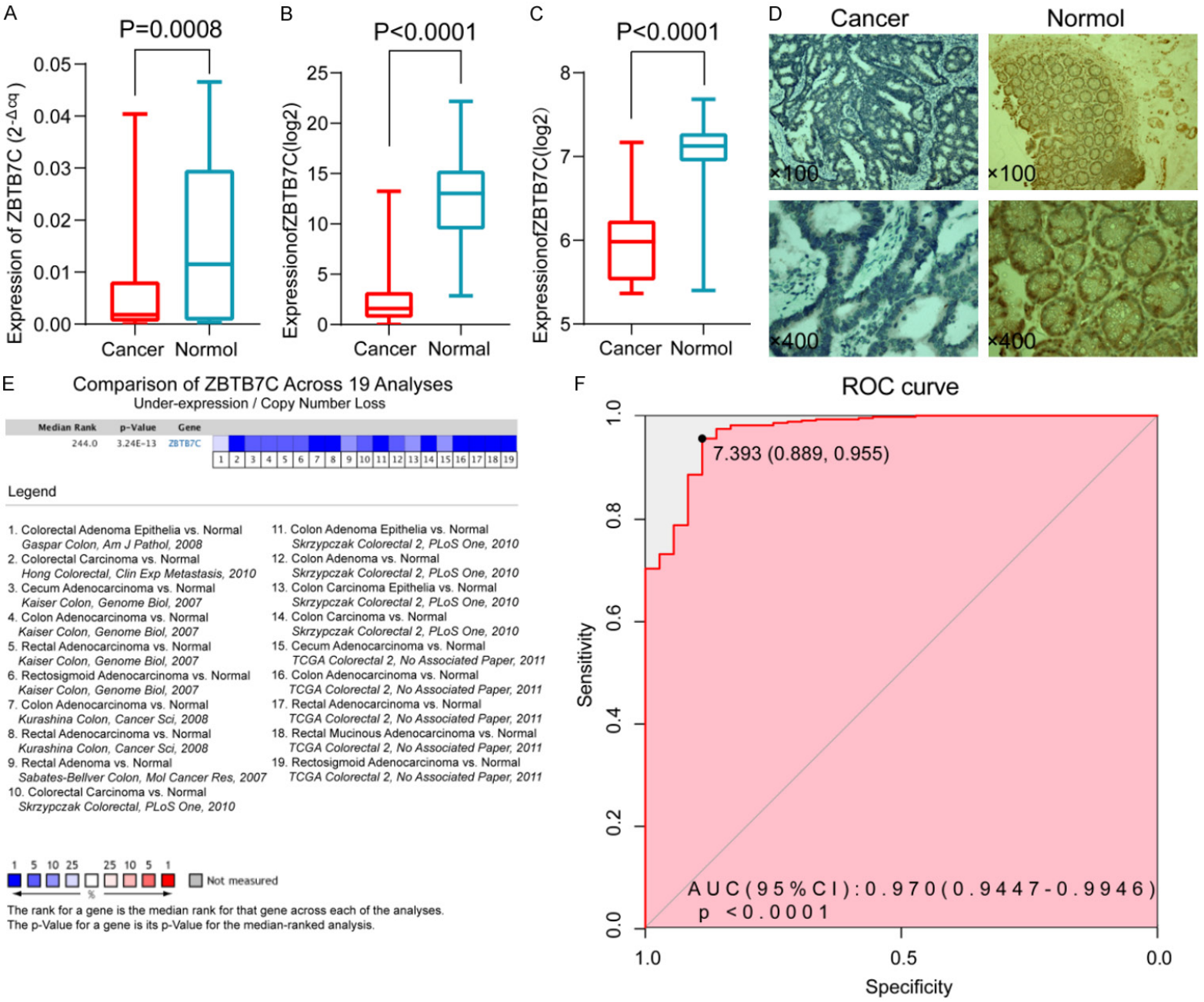

A
P=0.0008
Expression of ZBTB7C (2-Δcq)
0.05
0.04
0.03
0.02
0.01
0.00
Cancer
Normol
B
P<0.0001
ExpressionofZBTB7C(log2)
25
20
15
10
5
0
Cancer
Normal
C
P<0.0001
ExpressionofZBTB7C(log2)
8
7
6
5
Cancer
Normol
D
Cancer
Normol
×100
×100
×400
×400
E
Comparison of ZBTB7C Across 19 Analyses
Under-expression / Copy Number Loss
Median Rank
p-Value
Gene
244.0
3.24E-13
ZBTB7C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Legend
1. Colorectal Adenoma Epithelia vs. Normal
Gaspar Colon, Am J Pathol, 2008
2. Colorectal Carcinoma vs. Normal
Hong Colorectal, Clin Exp Metastasis, 2010
3. Cecum Adenocarcinoma vs. Normal
Kaiser Colon, Genome Biol, 2007
4. Colon Adenocarcinoma vs. Normal
Kaiser Colon, Genome Biol, 2007
5. Rectal Adenocarcinoma vs. Normal
Kaiser Colon, Genome Biol, 2007
6. Rectosigmoid Adenocarcinoma vs. Normal
Kaiser Colon, Genome Biol, 2007
7. Colon Adenocarcinoma vs. Normal
Kurashina Colon, Cancer Sci, 2008
8. Rectal Adenocarcinoma vs. Normal
Kurashina Colon, Cancer Sci, 2008
9. Rectal Adenoma vs. Normal
Sabates-Bellver Colon, Mol Cancer Res, 2007
10. Colorectal Carcinoma vs. Normal
Skrzypczak Colorectal, PLoS One, 2010
11. Colon Adenoma Epithelia vs. Normal
Skrzypczak Colorectal 2, PLoS One, 2010
12. Colon Adenoma vs. Normal
Skrzypczak Colorectal 2, PLoS One, 2010
13. Colon Carcinoma Epithelia vs. Normal
Skrzypczak Colorectal 2, PLoS One, 2010
14. Colon Carcinoma vs. Normal
Skrzypczak Colorectal 2, PLoS One, 2010
15. Cecum Adenocarcinoma vs. Normal
TCGA Colorectal 2, No Associated Paper, 2011
16. Colon Adenocarcinoma vs. Normal
TCGA Colorectal 2, No Associated Paper, 2011
17. Rectal Adenocarcinoma vs. Normal
TCGA Colorectal 2, No Associated Paper, 2011
18. Rectal Mucinous Adenocarcinoma vs. Normal
TCGA Colorectal 2, No Associated Paper, 2011
19. Rectosigmoid Adenocarcinoma vs. Normal
TCGA Colorectal 2, No Associated Paper, 2011
1 5 10 25
25 10 5 1
%
Not measured
The rank for a gene is the median rank for that gene across each of the analyses.
The p-Value for a gene is its p-Value for the median-ranked analysis.
F
ROC curve
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
7.393 (0.889, 0.955)
AUC(95%CI):0.970(0.9447-0.9946)
p <0.0001
1.0
0.5
0.0
Specificity

Figure 2. A-C. ZBTB7C expression between CRC tissues and paracancerous tissues. A. 27 paired CRC tissues and paracancerous tissues from the First Affiliated Hospital of Chongqing Medical University. B. From TCGA. C. From GEO. D. Representative images of ZBTB7C expression in paracancerous tissues and CRC tissues (100× and 400×) detected by IHC staining. E. Studies on the expression of ZBTB7C in CRC are labeled 1-19; blue represents decreased expression, with deeper colors indicating increased expression. F. ROC curve of ZBTB7C expression in CRC tissue.

Table 1. Immunohistochemistry assay for ZBTB7C protein expression in CRC tissues and normal colorectal tissues

Tissues	N	ZBTB7C protein expression		χ^2	P
		+	-		
CRC	69	30	39	50.835	0.000
Normal	69	68	1		

Abbreviations: CRC, colorectal cancer; ZBTB7C, zinc finger and BTB domain containing 7C.

prognosis for patients with CRC. Furthermore, this conclusion was also validated by the GEPIA (P=0.013, **Figure 3B**) and OncoLnc (P=0.0158, **Figure 3C**) analyses.

Next, we examined the potential of ZBTB7C expression as a prognostic biomarker for CRC by performing a Cox proportional hazard regression analysis. According to the univariate analysis, a younger age, early TNM stage and high ZBTB7C expression were predictors of an extended overall survival. In the multivariate analysis, ZBTB7C was an independent prognostic factor for CRC as a tumor suppressor gene (hazard ratio =0.516; 95% confidence interval (CI), 0.302-0.880; P=0.015; **Table 3**).

Biological pathways associated with the ZBTB7C gene

GSEA was used to map ZBTB7C in the BioCarta pathway database and predict the possible biological pathways associated with ZBTB7C. According to the cut-off criteria of $P < 0.05$, gene size ≥ 15, FDR < 0.25, and $|ES| > 0.5$, samples in the high expression group were enriched for ZBTB7C in multiple metabolism-related datasets (n=13), suggesting that ZBTB7C may participate in multiple biological processes involved in CRC (**Figure 4**).

Bioinformatics analyses

GO and KEGG analyses were implemented with the 349 genes using the clusterProfiler R package and KOBAS 3.0. PPI networks were analyzed using STRING. The GO analysis identified coexpressed genes that mainly participated in biological processes such as O-glycan processing, glycosylation, and glycoprotein metabolic processes; the main cellular components included the nuclear periphery, nuclear transcription factor complex, and transcription factor complex; and the main molecular functions were proximal promoter sequence-specific DNA binding, RNA polymerase II proximal promoter sequence-specific DNA binding, and chromatin binding (**Figure 5** and Table S1). The KEGG pathway analysis identified 21 signaling pathways with which ZBTB7C may be associated. The most important pathways were metabolic pathways, pathways in cancer, and the Ras signaling pathway (**Figure 6** and Table S2). After removing the unconnected nodes, we established a PPI network that contained 348 nodes and 402 edges (**Figure 7**), and the top 10 genes are listed in Table S3. The hub genes in the network play critical roles in the process of CRC; therefore, the closeness centrality was evaluated, and the cytoHubba app was used to extract the following top 10 hub genes: MYC, FOXA1, POLR2A, NCOA3, UBE2C, RUVBL1, E2F1, KAT2A, HSP-90AB1 and CEBPB (**Figure 8A** and Table S4).

Prediction and meta-analysis of ZBTB7C target miRNAs

The top five target miRNAs that passed the target prediction criteria were included in our study. This meta-analysis included 8 GEO datasets and TCGA databases, and the expression of the five miRNAs in CRC tissues was verified. The expression of hsa-miR-452-5p was increased in CRC tissues (P=0.033) (**Figure 8B**). Moreover, hsa-miR-452-5p and ZBTB7C have one complementary sequence fragment: 3'-UUUGUCA-5'-3'-UGACAAA-5' (**Figure 8C**). This fragment may be a potential target. In addition, the diagnostic value of miR-452-5p in CRC was clarified using the sROC method. Based on this result, hsa-miR-452-5p has a good differential diagnostic value in CRC, with an AUC of 0.92 (95% CI, 0.90, 0.94) (**Figure 8D**), an optimal

Table 2. Correlation between ZBTB7C and clinicopathological parameters of colorectal cancer patients

Clinicopathological parameters	Cases (N)	ZBTB7C expression levels		χ^2	*P* value
		Low (%)	High (%)		
Age (years)					
< 60	116	56 (48.3)	60 (51.7)	0.165	0.685
≥ 60	309	156 (50.5)	153 (49.5)		
Gender					
Male	227	114 (50.2)	113 (49.8)	0.022	0.881
Female	198	98 (49.5)	100 (50.5)		
T stage					
1	12	4 (33.3)	8 (66.7)	4.779	0.189
2	79	47 (59.5)	32 (40.5)		
3	292	142 (48.6)	150 (51.4)		
4	42	19 (45.2)	23 (54.8)		
N stage					
0	257	119 (46.3)	138 (53.7)	4.754	0.093
1	99	51 (51.5)	48 (48.5)		
2	69	42 (60.9)	27 (39.1)		
M stage					
0	357	172 (48.2)	185 (51.8)	2.589	0.108
1	68	40 (58.8)	28 (41.2)		
TMN stage					
I	81	43 (53.1)	38 (46.9)	6.186	0.103
II	168	72 (42.9)	96 (57.1)		
III	108	57 (52.8)	51 (47.2)		
IV	68	40 (58.8)	28 (41.2)		

Abbreviation: ZBTB7C, zinc finger and BTB domain containing 7C.

sensitivity of 0.84 (95% CI, 0.51, 0.96) and a specificity of 0.87 (95% CI, 0.59, 0.97).

Discussion

This study used basic experiments combined with bioinformatics methods to show for the first time that ZBTB7C expression was significantly downregulated in CRC tissues at both the mRNA and protein levels. The ROC curve confirmed that ZBTB7C is an excellent diagnostic marker for CRC. We subsequently explored the important role of ZBTB7C in determining the prognosis of patients with CRC. ZBTB7C functions as a tumor suppressor gene, and higher expression predicts a better overall survival. Moreover, ZBTB7C expression was not associated with other clinicopathological parameters. In other words, it does not affect the prognosis by altering other well-defined pathological factors. The multivariate Cox regression analysis confirmed that the gene is an independent prognostic factor for CRC. In addition, the TCGA data indicated genetic alterations in ZBTB7C in 6% of CRC tissues. The GSEA indicated that ZBTB7C is associated with multiple metabolic pathways. Because of the complexity and polygenicity of the tumor in which changes in a single gene are not sufficiently powerful to cause the tumor, we focused on the genes coexpressed with ZBTB7C. By conducting a bioinformatics analysis of these coexpressed genes, we identified a potentially protective role for ZBTB7C in CRC by participating in the regulation of the Ras signaling pathway and the Wnt signaling pathway. In addition, preliminary validation by miRWalk2.0 prediction and meta-analysis confirmed that miR-452-5p is expressed at high levels in CRC tissues and contains potential complementary target sequences to ZBTB7C.

The differential expression of ZBTB7C and its biological pathways involved in regulating tu-

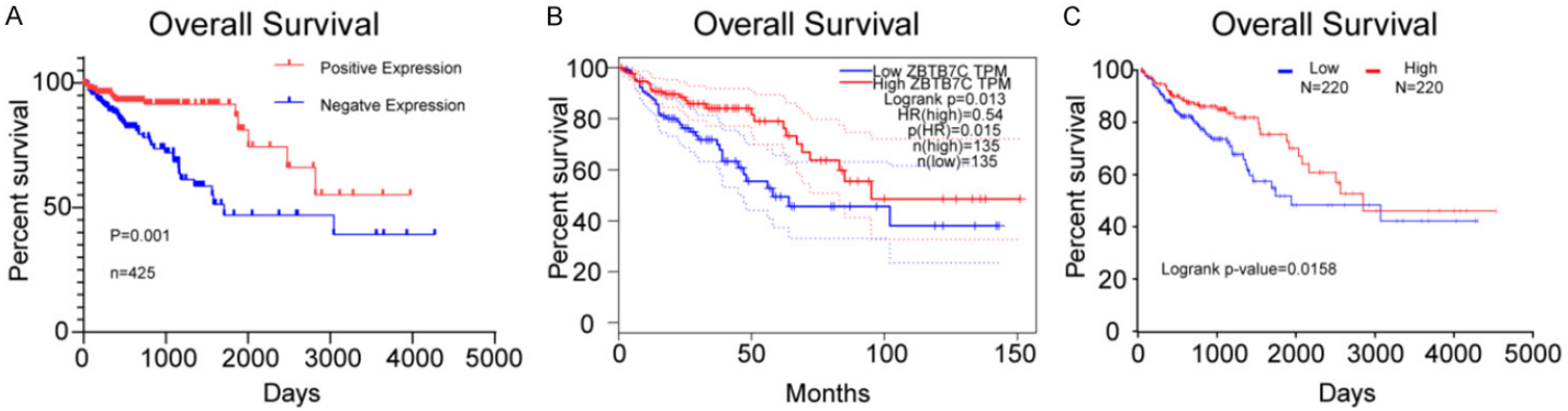


Figure 3. A-C. Kaplan-Meier analysis of overall survival in colorectal cancer patients with high or low ZBTB7C expression. A. TCGA. B. GEPIA. C. OncoLnc. We found that the overall survival (OS) was lower in the ZBTB7C low expression group (blue) than in the high expression group (red). Therefore, our data suggest that ZBTB7C may be a potential target for the prognosis of CRC.

Table 3. Prognostic factors for overall survival of CRC patients estimated by univariate and multivariate Cox regression analyses

Characteristics	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (years)						
< 60	1	-	-	1	-	-
≥ 60	1.972	1.032-3.768	0.040	2.454	1.258-4.786	0.008
Gender						
Male	1	-	-	-	-	-
Female	0.949	0.587-1.535	0.832	-	-	-
T stage						
1	1	-	-	1	-	-
2	0.871	0.097-7.809	0.902	0.174	0.011-2.801	0.217
3	2.225	0.306-16.183	0.430	0.199	0.009-4.214	0.300
4	8.006	1.059-60.549	0.044	0.397	0.018-8.753	0.558
N stage						
0	1	-	-	1	-	-
1	2.842	1.562-5.173	0.001	0.796	0.171-3.709	0.771
2	4.975	2.779-8.909	< 0.0001	0.962	0.197-4.692	0.962
M stage						
0	1	-	-	-	-	-
1	5.326	3.273-8.667	< 0.0001	-	-	-
TNM stage						
I	1	-	-	1	-	-
II	3.206	0.736-13.966	0..121	4.319	0.395-47.190	0.230
III	6.149	1.426-26.511	0.015	9.893	0.750-130.551	0.082
IV	19.275	4.602-80.736	< 0.0001	26.373	2.132-326.208	0.011
ZBTB7C expression						
Low	1	-	-	1	-	-
High	0.420	0.252-0.699	0.001	0.516	0.302-0.880	0.015

Abbreviation: CRC, colorectal cancer; ZBTB7C, zinc finger and BTB domain containing 7C.

mor growth have been preliminary studied in other tumors. However, these findings were not sufficient to conclusively determine the role of ZBTB7C in tumors and require further refinement by our group and other researchers. On the one hand, the gene is expressed as a proto-oncogene and a tumor suppressor gene in different tumors; on the other hand, it indirectly affects the metabolism of tumor cells and the occurrence and development of tumors. Studies by Hurr and Jeon have shown high expression of ZBTB7C in renal cell carcinoma [10-12]. Lee noted that ZBTB7C was overexpressed in breast, kidney, brain and bone marrow cancers [13]. However, an opposite result was reported in HeLa and CaSki cervical carcinoma cell lines by Reuter and colleagues [9]. These differences in function and expression suggest that ZBTB7C is a tissue-specific gene that may be involved in multiple pathways and differentially regulated in different tumors. Currently, no study has described the relationship between ZBTB7C and CRC. Therefore, this experiment confirmed the low expression of ZBTB7C in CRC tissues using RT-qPCR, immunohistochemistry, and the GEO and TCGA databases.

The Kaplan-Meier survival curve revealed prolonged overall survival of patients with CRC presenting high ZBTB7C expression. The Cox regression analysis identified ZBTB7C as an independent prognostic factor for CRC. Notably, due to the incomplete TCGA data, this study was unable to consider the effects of different ethnicities on the outcomes. At the same time, verification experiments were not performed

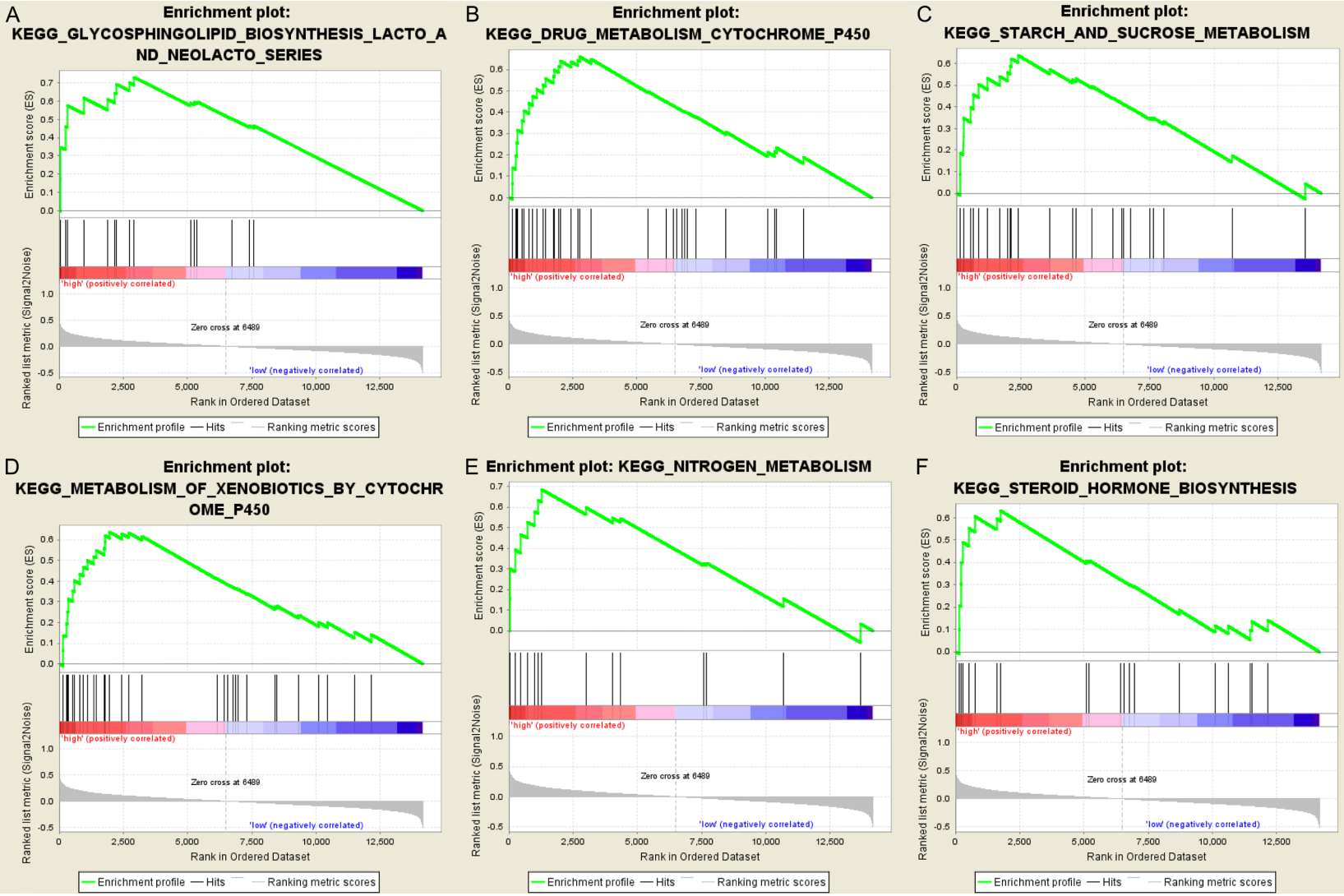
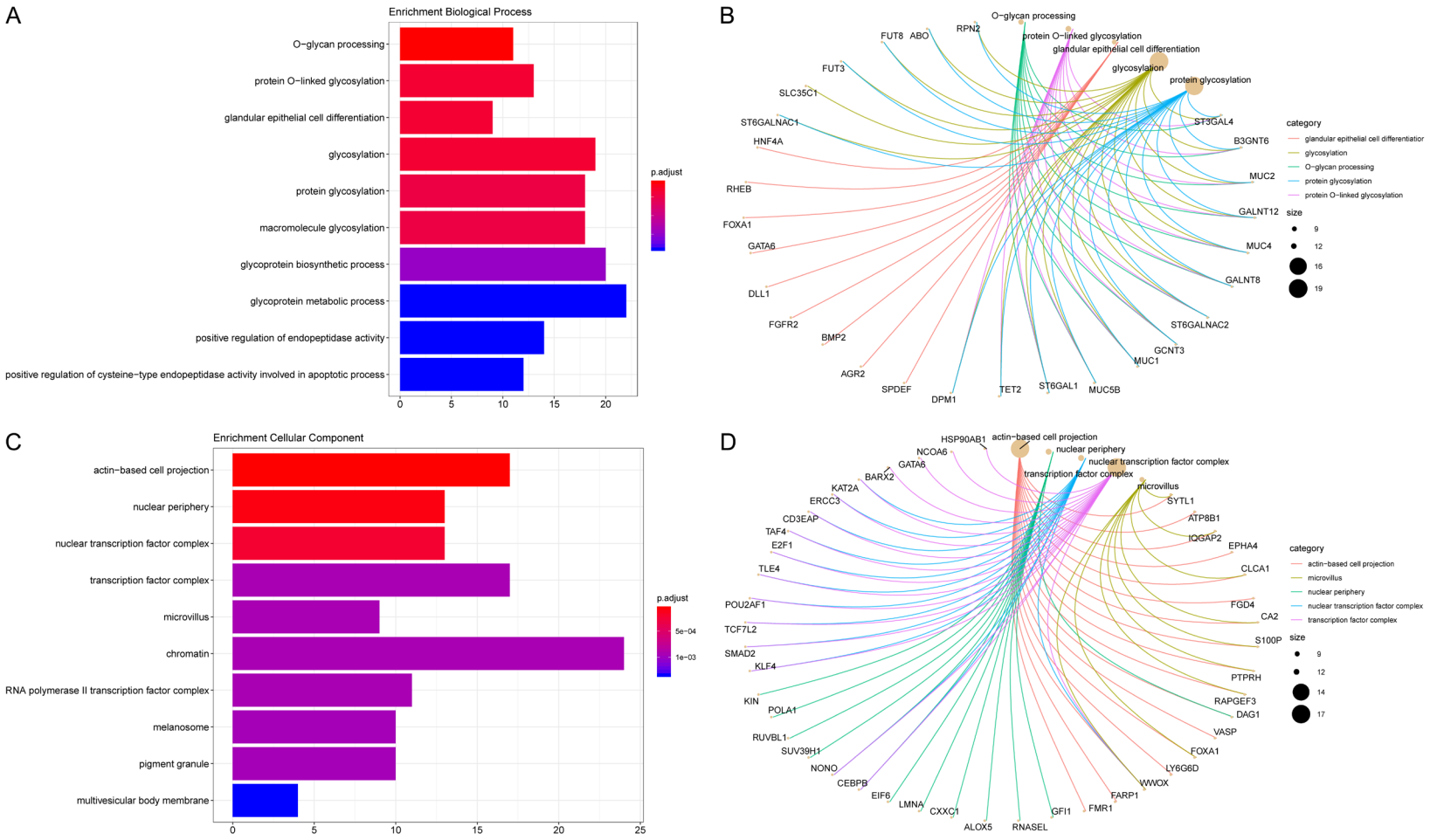


Figure 4. The six most common functional gene sets enriched in CRC samples with high ZBTB7C expression are listed. We found that the pathway of ZBTB7C mainly focused on metabolism related pathway. It may affect the occurrence and development of tumor by affecting the glycometabolism and lipid metabolism.


A
Enrichment Biological Process
O−glycan processing
protein O−linked glycosylation
glandular epithelial cell differentiation
glycosylation
protein glycosylation
macromolecule glycosylation
glycoprotein biosynthetic process
glycoprotein metabolic process
positive regulation of endopeptidase activity
positive regulation of cysteine−type endopeptidase activity involved in apoptotic process
0
5
10
15
20
p.adjust
B
O−glycan processing
RPN2
FUT8
ABO
protein O−linked glycosylation
glandular epithelial cell differentiation
glycosylation
protein glycosylation
FUT3
SLC35C1
ST6GALNAC1
HNF4A
RHEB
FOXA1
GATA6
DLL1
FGFR2
BMP2
AGR2
SPDEF
DPM1
TET2
ST6GAL1
MUC5B
MUC1
GCNT3
ST6GALNAC2
GALNT8
MUC4
GALNT12
MUC2
B3GNT6
ST3GAL4
category
glandular epithelial cell differentiation
glycosylation
O−glycan processing
protein glycosylation
protein O−linked glycosylation
size
9
12
16
19
C
Enrichment Cellular Component
actin−based cell projection
nuclear periphery
nuclear transcription factor complex
transcription factor complex
microvillus
chromatin
RNA polymerase II transcription factor complex
melanosome
pigment granule
multivesicular body membrane
0
5
10
15
20
25
p.adjust
5e−04
1e−03
D
HSP90AB1
actin−based cell projection
NCOA6
nuclear periphery
GATA6
nuclear transcription factor complex
BARX2
transcription factor complex
KAT2A
microvillus
ERCC3
SYTL1
CD3EAP
ATP8B1
TAF4
IQGAP2
E2F1
EPHA4
TLE4
CLCA1
POU2AF1
FGD4
TCF7L2
CA2
SMAD2
S100P
KLF4
PTPRH
KIN
RAPGEF3
POLA1
DAG1
RUVBL1
VASP
SUV39H1
FOXA1
NONO
LY6G6D
CEBPB
WWOX
EIF6
FARP1
LMNA
FMR1
CXXC1
GFI1
ALOX5
RNASEL
category
actin−based cell projection
microvillus
nuclear periphery
nuclear transcription factor complex
transcription factor complex
size
9
12
14
17

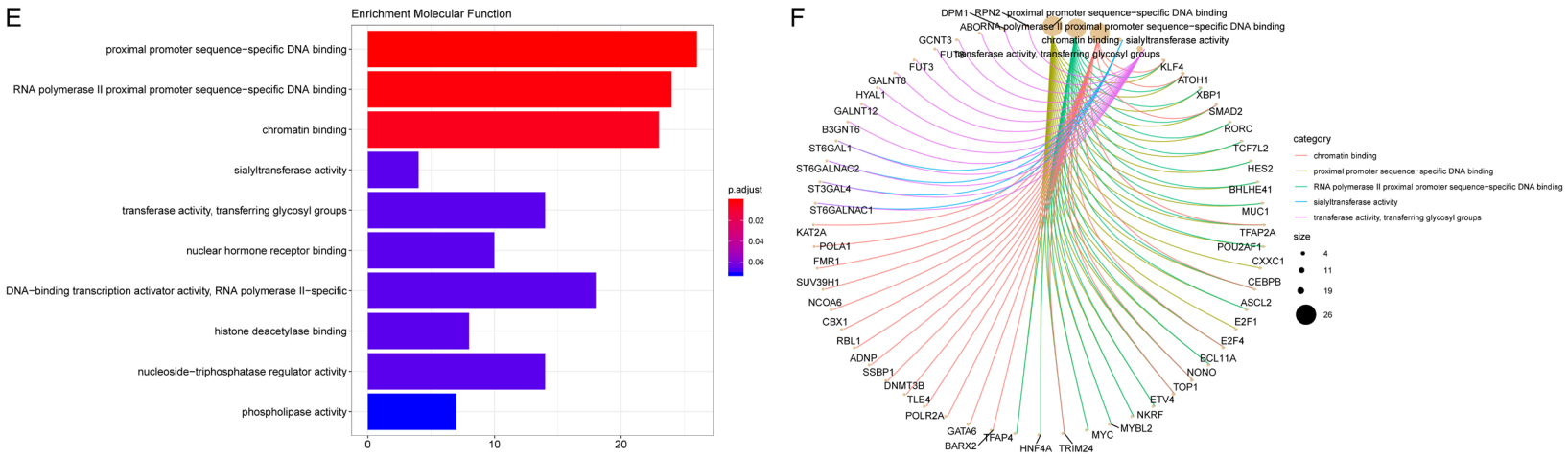


Figure 5. Enriched GO categories of the genes coexpressed with ZBTB7C. GO enrichment analysis was performed using the clusterProfiler method to show the biological processes (A and B), cellular components (C and D) and molecular functions (E and F). The length of the histogram represents the count number, and the color represents the adjust *p* value. The line color of the ring graph represents the GO terms, the points represent the genes, and the size of the points represents the count number.

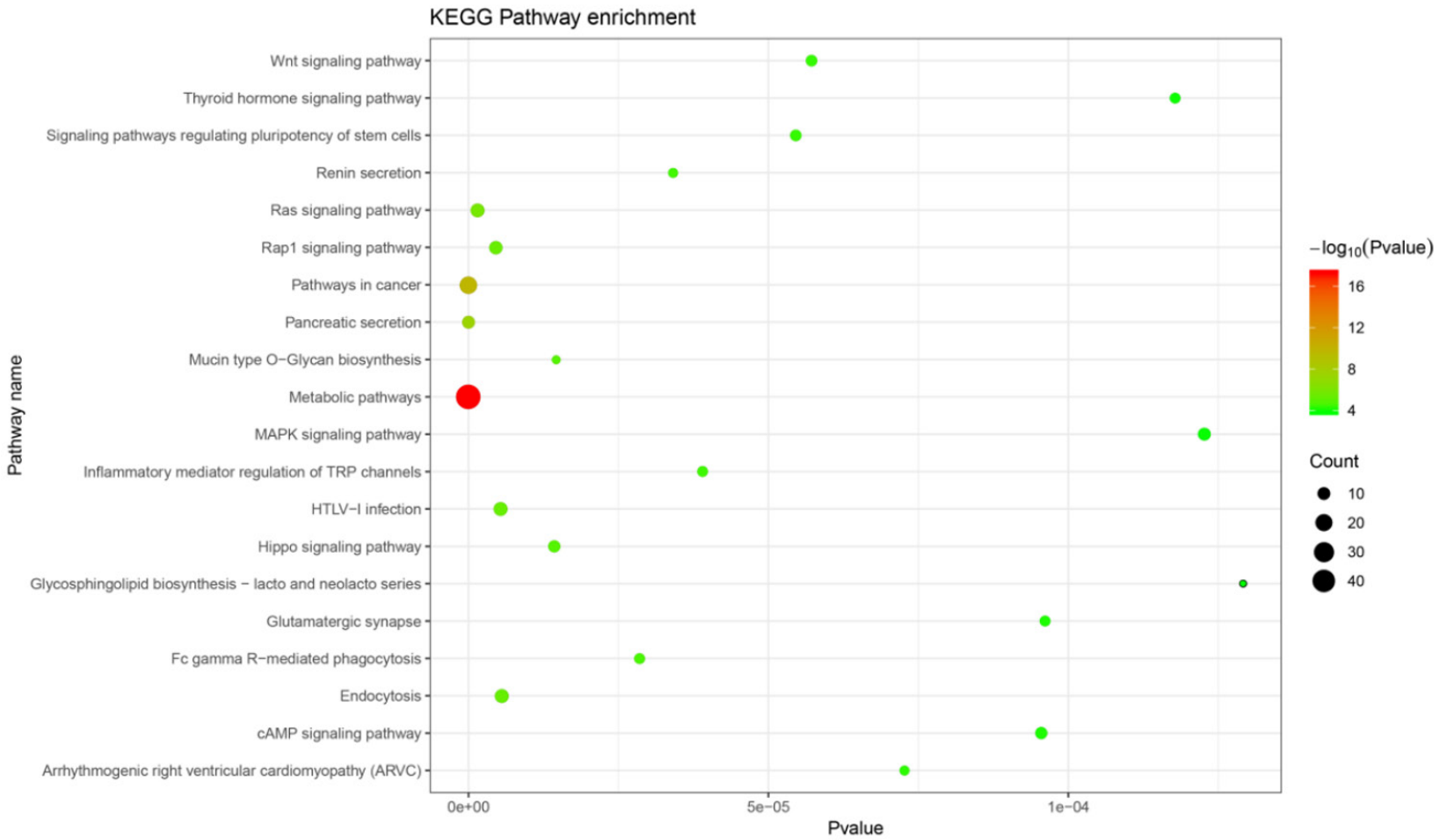


Figure 6. Pathway analysis using the KOBAS method based on the KEGG database of ZBTB7C coexpression genes in CRC. The x-axis represents the gene ratio, and the y-axis represents the KEGG terms. The size of the circle represents the gene count. The color of the circle represents different adjusted *p* values.

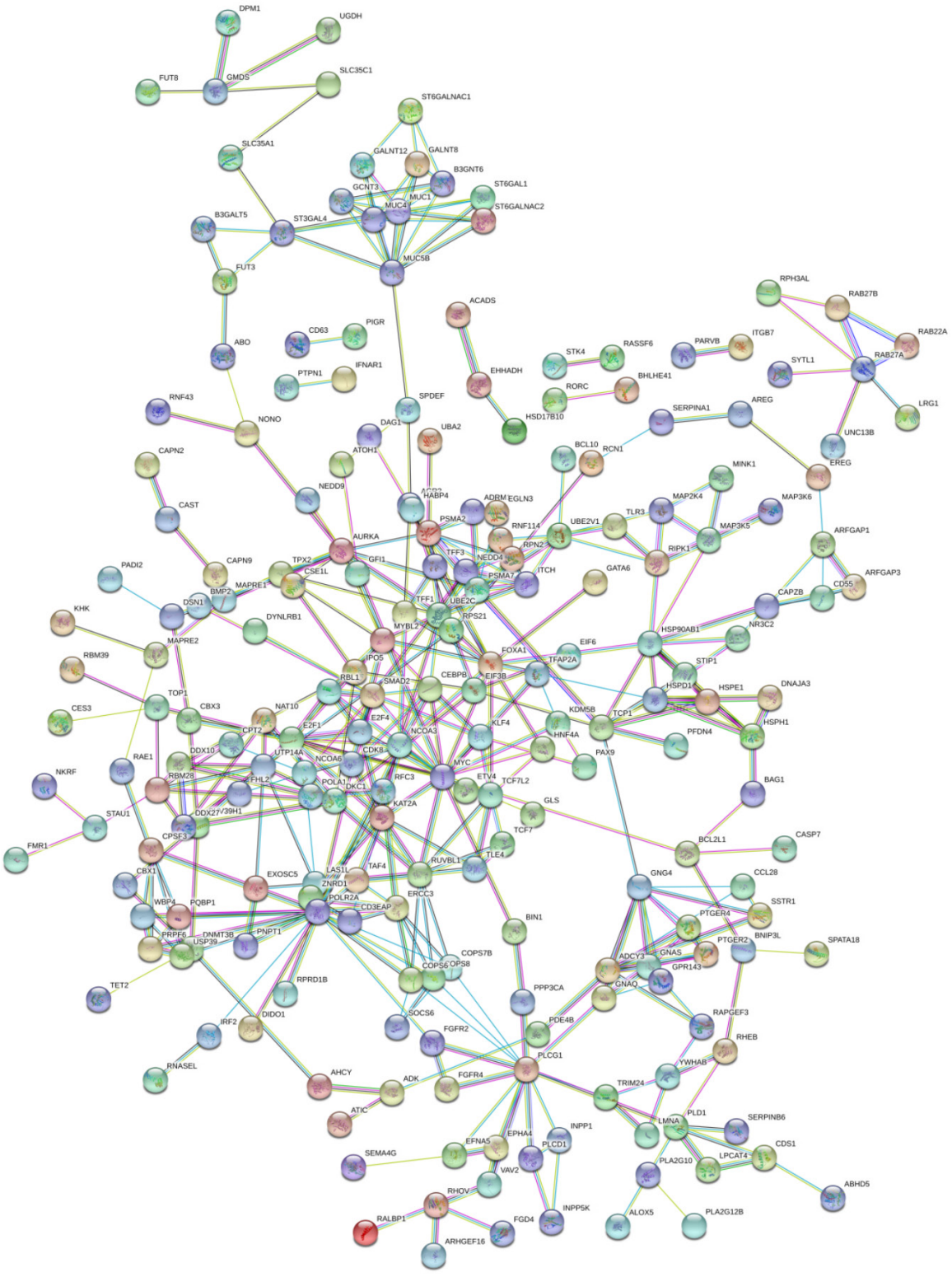
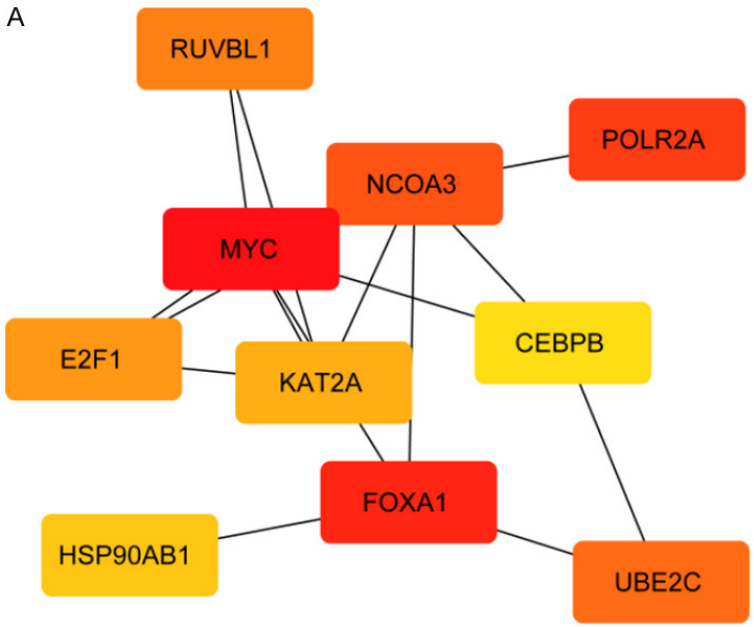
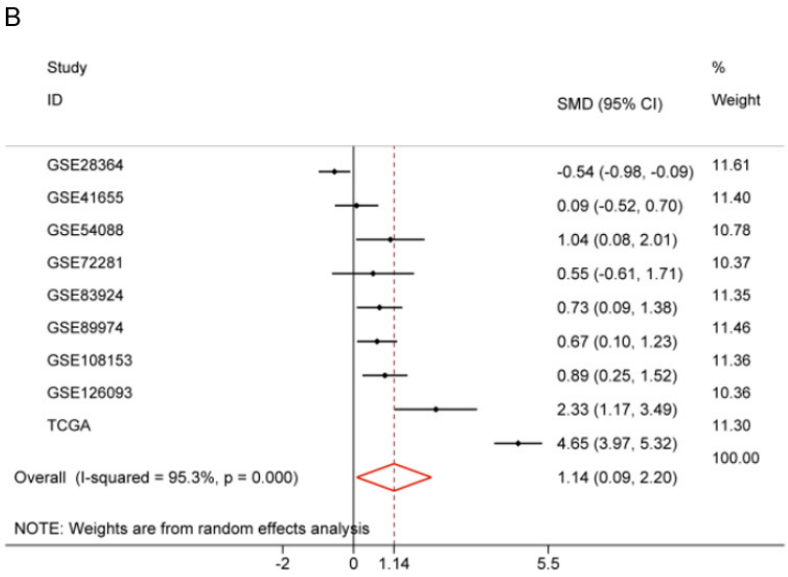


Figure 7. PPI network analysis of genes coexpressed in ZBTB7C in CRC. We set minimum required interaction score as 0.4 and hide disconnected nodes in the network. PPI network that contained 348 nodes and 402 edges.

for the comparisons. Therefore, a follow-up study is needed to verify these findings. Nevertheless, ZBTB7C expression is expected to be a new independent prognostic factor in addition to microsatellite instability [22], the BRAF mutation status [23], gene expression


A
RUVBL1
POLR2A
NCOA3
MYC
E2F1
KAT2A
CEBPB
FOXA1
HSP90AB1
UBE2C


B
Study
ID
SMD (95% CI)
%
Weight
GSE28364
GSE41655
GSE54088
GSE72281
GSE83924
GSE89974
GSE108153
GSE126093
TCGA
Overall (I-squared = 95.3%, p = 0.000)
-0.54 (-0.98, -0.09)
0.09 (-0.52, 0.70)
1.04 (0.08, 2.01)
0.55 (-0.61, 1.71)
0.73 (0.09, 1.38)
0.67 (0.10, 1.23)
0.89 (0.25, 1.52)
2.33 (1.17, 3.49)
4.65 (3.97, 5.32)
1.14 (0.09, 2.20)
11.61
11.40
10.78
10.37
11.35
11.46
11.36
10.36
11.30
100.00
NOTE: Weights are from random effects analysis
-2
0
1.14
5.5


C
5' ucuggccuauuuauuAAACAGUa Position 2354-2361 of ZBTB7C 3' UTR
3' agucaaaggagacg UUUGUCAa hsa-miR-452-5p

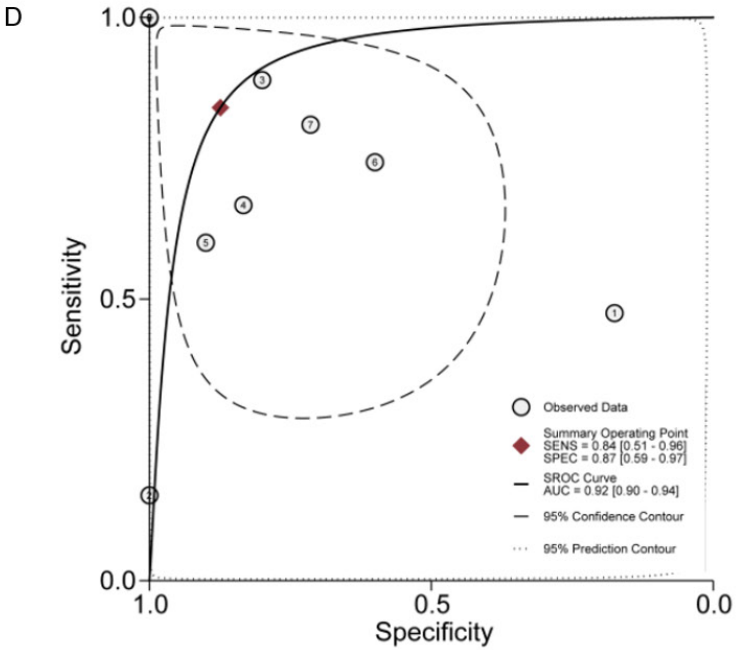

D
1.0
0.5
0.0
Sensitivity
1.0
0.5
0.0
Specificity
Observed Data
Summary Operating Point
SENS = 0.84 [0.51 - 0.96]
SPEC = 0.87 [0.59 - 0.97]
SROC Curve
AUC = 0.92 [0.90 - 0.94]
95% Confidence Contour
95% Prediction Contour

Figure 8. A. The most significant module was obtained from the PPI network. The redder the color, the higher the ranking. B. Forest plot of miR-452-5p between CRC and paracancerous tissues. C. The complementary sequences of miR-452-5p and ZBTB7C. D. The sROC curve of miR-452-5p expression between CRC and paracancerous tissues.

profile [24] and polygenic mutation characteristics [25].

As a complex transcription factor, ZBTB7C regulates multiple pathways. Both the GSEA and KEGG results showed that ZBTB7C is mainly enriched in metabolic pathways and pathways in cancer. On the one hand, according to previous studies, this transcription factor affects fatty acid metabolism in tumor cells and thus alters the cell cycle of tumor cells [10, 11]. However, depending on the enrichment results, the affected metabolic pathways of tumor cells may also include glucose metabolism and cytochrome P450-related metabolism. On the other hand, two main pathways were identified to explain the role of ZBTB7C in CRC: the Ras signaling pathway and the Wnt signaling pathway. Recent studies have reported the abnormal activation of the Ras pathway in many malignant tumor tissues; for example, McCubrey et al. [26] detected Ras mutations in a variety of tumors, such as melanoma, colon cancer, and ovarian cancer. The Ras protein functions as a tumor suppressor [27], and its single point mutation is sufficient to trigger malignant transformation [28, 29]. Recently, RAS was shown to be a potential biological target of CRC [29]. The Wnt family participates in an important cellular signaling pathway regulates cell proliferation, differentiation, and migration. The Wnt signaling pathway is closely related to the development of various tumors, such as CRC [30], gastric cancer [31], lung cancer [32], cervical cancer [33], and malignant melanoma [34]. These two pathways play a pivotal role in a variety of tumors.

In summary, ZBTB7C may cooperate with coexpressed genes such as MYC and RUVBL1 to regulate the Ras signaling pathway and the Wnt signaling pathway, thereby functioning in CRC. At the same time, it may also alter the metabolic pathways involved in tumor cells. These predictions derived from bioinformatics analyses require functional tests for verification.

Finally, based on online software and a meta-analysis, we confirmed that miR-452-5p is upregulated in CRC, in contrast to ZBTB7C expression. Based on previous studies, miR-452-5p is expressed at high levels in breast cancer, lung squamous cell carcinoma and renal cancer. Notably, miR-452-5p promotes renal cell carcinoma (RCC) cell migration and invasion in vitro and in vivo [35-37]. Therefore, miR-452-5p may inhibit the expression of ZBTB7C in CRC tissues by targeting complementary sequences. However, specific inhibition methods (splicing the ZBTB7C mRNA, inhibiting ZBTB7C translation or inhibiting binding) require further study.

In summary, we conducted a comprehensive analysis of ZBTB7C expression in CRC. This study is the first to report low expression of ZBTB7C in CRC and its association with a poor prognosis. At the same time, the potential of ZBTB7C expression to serve as a differential diagnostic factor and an independent prognostic factor has also been confirmed. Furthermore, ZBTB7C may regulate the Ras signaling pathway and the Wnt signaling pathway by affecting coexpressed genes (e.g., MYC and RUVBL1) or may be associated with miR-452-5p. This study provides a comprehensive and reliable theoretical basis and research data for subsequent studies of ZBTB7C in CRC.

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Disclosure of conflict of interest

None.

Address correspondence to: Zheng Jiang, Department of Gastroenterology, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Chongqing 400016, P. R. China. E-mail: zhengj17-53@126.com

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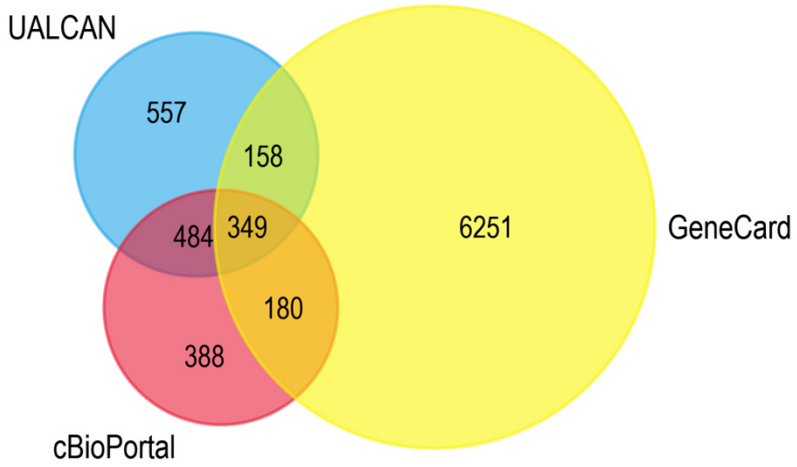

Figure S1. Genes coexpressed with ZBTB7C. Based on the UALCAN database, 1548 strongly coexpressed genes were acquired. Similarly, cBioPortal identified 1401 coexpressed genes in the CRC group. In addition, 6938 CRC-related genes were identified in GeneCards. Finally, the results obtained from UALCAN, cBioPortal and GeneCards were analyzed for common genes, and 349 genes were identified.

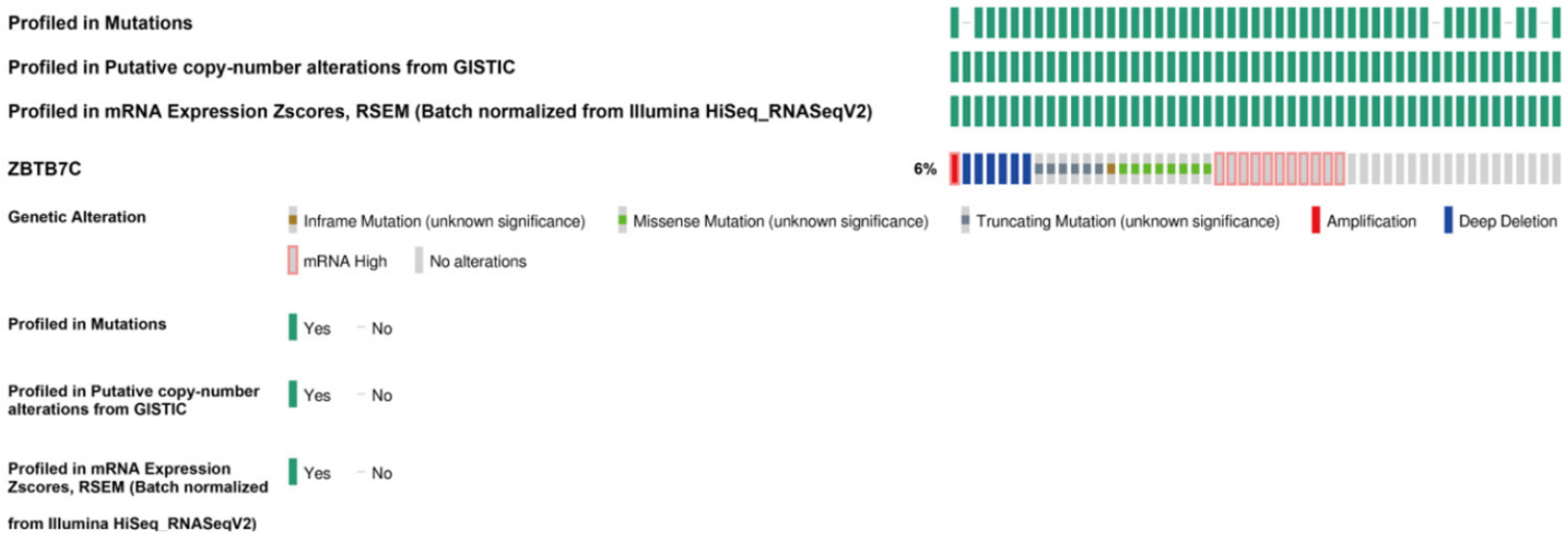

Figure S2. The OncoPrint schematic revealed that the ZBTB7C gene was altered in 33/594 (6%) sequenced samples, with 1 case of amplification, 8 cases of missense mutations, 6 cases of deep deletions, 11 cases of mRNA upregulation, 1 case of an inframe mutation and 6 cases of truncating mutations.

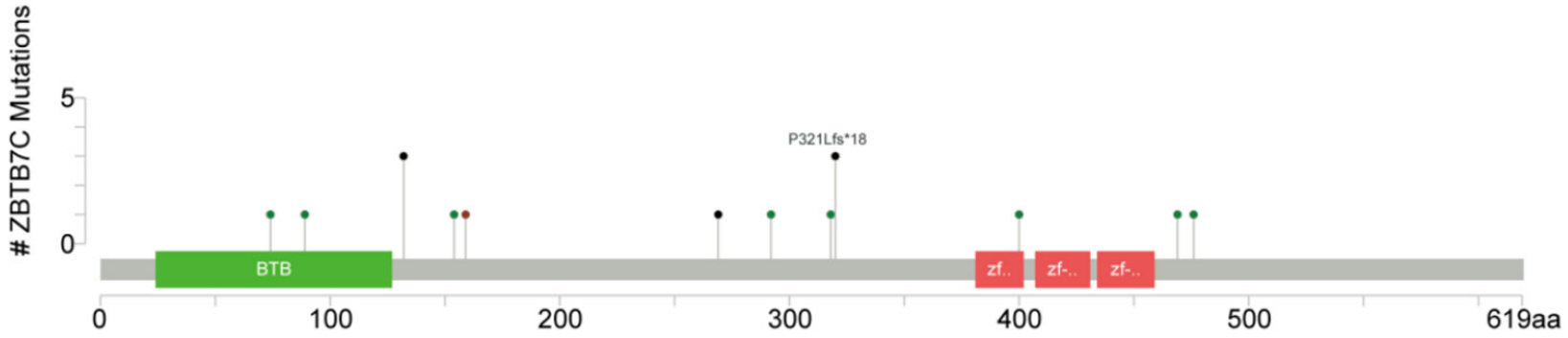

Figure S3. The ZBTB7C mutation locations were identified with OncoPrint, the somatic mutation frequency of ZBTB7C was 2.9%, and missense mutations were the most common type of mutation in CRC.

Table S1. The top ten most significant items of Gene Ontology (GO) terms of the co-expression genes of Zinc finger and BTB domain containing 7C (ZBTB7C) in colorectal cancer

Category	Term	Count	*P*-value
Biological processes			
GO:0016266	O-glycan processing	11	5.49E-05
GO:0006493	protein O-linked glycosylation	13	0.000168611
GO:0002067	glandular epithelial cell differentiation	9	0.000168611
GO:0070085	glycosylation	19	0.000168611
GO:0006486	protein glycosylation	18	0.000233509
GO:0043413	macromolecule glycosylation	18	0.000233509
GO:0009101	glycoprotein biosynthetic process	20	0.000736993
GO:0009100	glycoprotein metabolic process	22	0.00095043
GO:0010950	positive regulation of endopeptidase activity	14	0.00095043
GO:0043280	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	12	0.00095043
Cellular components			
GO:0098858	actin-based cell projection	17	5.90E-05
GO:0034399	nuclear periphery	13	9.87E-05
GO:0044798	nuclear transcription factor complex	13	0.000220775
GO:0005667	transcription factor complex	17	0.0009361
GO:0005902	microvillus	9	0.000939454
GO:0000785	chromatin	24	0.000939454
GO:0090575	RNA polymerase II transcription factor complex	11	0.000939454
GO:0042470	melanosome	10	0.000960593
GO:0048770	pigment granule	10	0.000960593
GO:0032585	multivesicular body membrane	4	0.001345567
Molecular functional			
GO:0000987	proximal promoter sequence-specific DNA binding	26	0.000901429
GO:0000978	RNA polymerase II proximal promoter sequence-specific DNA binding	24	0.00207221
GO:0003682	chromatin binding	23	0.005074812
GO:0008373	sialyltransferase activity	4	0.066568782
GO:0016757	transferase activity, transferring glycosyl groups	14	0.066568782
GO:0035257	nuclear hormone receptor binding	10	0.066568782
GO:0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	18	0.066568782
GO:0042826	histone deacetylase binding	8	0.066568782
GO:0060589	nucleoside-triphosphatase regulator activity	14	0.066568782
GO:0004620	phospholipase activity	7	0.071954252

Table S2. KEGG pathways enriched by co-expression genes of Zinc finger and BTB domain containing 7C (ZBTB7C) mRNA in colorectal cancer

Category	Term	Count	*P*-value
KEGG_PATHWAY	Metabolic pathways	49	5.67E-18
KEGG_PATHWAY	Pathways in cancer	21	1.69E-10
KEGG_PATHWAY	Pancreatic secretion	10	3.33E-08
KEGG_PATHWAY	Ras signaling pathway	12	1.53E-06
KEGG_PATHWAY	Rap1 signaling pathway	11	4.55E-06
KEGG_PATHWAY	HTLV-I infection	12	5.34E-06
KEGG_PATHWAY	Endocytosis	12	5.55E-06
KEGG_PATHWAY	Hippo signaling pathway	9	1.43E-05
KEGG_PATHWAY	Mucin type O-Glycan biosynthesis	5	1.46E-05
KEGG_PATHWAY	Fc gamma R-mediated phagocytosis	7	2.85E-05
KEGG_PATHWAY	Renin secretion	6	3.41E-05
KEGG_PATHWAY	Inflammatory mediator regulation of TRP channels	7	3.90E-05
KEGG_PATHWAY	Signaling pathways regulating pluripotency of stem cells	8	5.46E-05

KEGG_PATHWAY	Wnt signaling pathway	8	5.72E-05
KEGG_PATHWAY	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	6	7.27E-05
KEGG_PATHWAY	cAMP signaling pathway	9	9.55E-05
KEGG_PATHWAY	Glutamatergic synapse	7	9.61E-05
KEGG_PATHWAY	Thyroid hormone signaling pathway	7	0.000117812
KEGG_PATHWAY	MAPK signaling pathway	10	0.000122673
KEGG_PATHWAY	Glycosphingolipid biosynthesis - lacto and neolacto series	4	0.00012913

Table S3. Top 10 with combined score co-expression correlation by STRING 11.0 of Zinc finger and BTB domain containing 7C (ZBTB7C) co-expression genes in colorectal cancer

#Node1	Node2	Node1 STRING Internal ID	Homology	Co-expression	Experimentally determined interaction	Automated text mining	Combined score
HSP90AB1	STIP1	4443770	0	0.939	0.831	0.665	0.999
COPS7B	COPS6	4444223	0	0.209	0.989	0.803	0.999
COPS8	COPS6	4441613	0	0.194	0.994	0.92	0.999
COPS7B	COPS8	4444223	0	0.108	0.966	0.771	0.999
TPX2	AURKA	4437665	0	0.952	0.982	0.886	0.999
HSPD1	HSPE1	4445814	0	0.963	0.969	0.988	0.999
PSMA7	PSMA2	4443629	0.881	0.987	0.992	0.676	0.999
EFNA5	EPHA4	4440049	0	0.188	0.962	0.926	0.999
E2F4	RBL1	4445201	0	0.145	0.827	0.98	0.999
MYC	KAT2A	4451407	0	0.084	0.993	0.731	0.999

Table S4. Top 10 hub genes in PPI network ranked by Closeness method

Rank	Name	Score
1	MYC	72.16785714
2	FOXA1	67.70119048
3	POLR2A	67.50039683
4	NCOA3	66.51785714
5	UBE2C	65.70119048
6	RUVBL1	64.04325397
7	E2F1	61.60515873
8	KAT2A	61.60039683
9	HSP90AB1	61.14166667
10	CEBPB	60.43849206