

MicroRNA-802 Suppresses Tumorigenesis of Colorectal Cancer via Regulating UBN2

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Background: The initiation and progression of colorectal cancer (CRC) are a multistep complex process regulated by multiple factors. Previous evidence indicated that microRNA-802 (miR-802) participated in tumorigenesis of numerous solid cancers; however, the potential roles and underlying mechanisms of miR-802 in CRC still need further exploration.

Methods: Quantitative real-time PCR (qRT-PCR) was employed to evaluate miR-802 levels in human CRC tissues and cell lines. In vitro proliferation, apoptosis, migration and invasion assays, and in vivo subcutaneous mouse xenograft model were utilized to examine the effects of miR-802 on the malignant behaviors of CRC cells. Then, bioinformatics prediction, dual-luciferase reporter, qRT-PCR, and Western blot was conducted to confirm the down-stream target of miR-802.

Results: MiR-802 was frequently down-regulated in CRC tissues and cells. Further analyses showed that the low expression of miR-802 in CRC tissues was significantly correlated with tumor progression and poor patients' prognosis. Overexpression of miR-802 profoundly inhibited proliferation, migration and invasion but promoted apoptosis of CRC cells, by contrast, miR-802 silencing exhibited opposite effects in vitro. Further animal experiment demonstrated that miR-802 could suppress tumor growth via inhibiting the proliferation and promoting the apoptosis of CRC cells in vivo. Mechanistically, miR-802 functioned as a tumor suppressor through inhibiting the expression of Ubinuclein-2 (UBN2) on post-transcriptional level. Moreover, upregulation of UBN2 expression could reverse the biological effects of CRC cells induced by miR-802 overexpression.

Conclusion: Our study demonstrates that miR-802 inhibits the proliferation, migration and invasion while promotes the apoptosis of CRC cells via directly suppressing UBN2 expression. These findings provide a promising biomarker and potential treatment target for CRC.

Keywords: colorectal cancer, miR-802, UBN2, proliferation, migration, invasion

Introduction

Colorectal cancer (CRC) remains an important digestive cancer and accounts for over 1.8 million newly diagnosed cases and 881,000 deaths in 2018, making it the third most common cancer but the second leading cause of cancer death worldwide.¹ With the progress of screening programs and different therapeutic methods including surgery, chemoradiotherapy, radiation therapy and immunotherapy strategies, the treatment effect of CRC patients has been improved over the past decades.^{2,3} In spite of this, the prognosis of late stage CRC remains poor, with only 20 months median survival time.^{3,4} Therefore, exploring novel biomarkers for early diagnosis and prognostic stratification, and discovering potential therapeutic targets are important to improve the clinical outcome of CRC.

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The occurrence and progression of CRC are a multistep complex process modulated by multiple factors, in which genetic and/epigenetic alterations play important role.^{3,5} MicroRNAs (miRNAs, miRs), as a cluster of endogenous non-coding RNAs of 18-24 nucleotides, have been demonstrated to play important role in CRC's development.^{6,7} Nowadays, emerging and increasing studies indicate that miRNAs can post-transcriptionally regulate downstream cancer-related genes expression via binding to the 3'-untranslated regions (3'-UTRs) of their messenger RNA (mRNA),⁸ thereby participating in the proliferation, apoptosis, migration, invasion, angiogenesis and immune escape of tumor cells.^{9,10} MiR-802, lied on human chromosome 21, has been demonstrated to be a cancer-related miRNA. To date, large amount of evidence has showed that aberrant miR-802 expression is involved in the progression of multiple solid cancers.¹¹⁻²⁰ Noteworthy, the biological role of miR-802 in the tumorigenesis of human cancer mainly depends on cancer type. Numerous studies reported that miR-802 functions as a tumor suppressor in most cancers, including glioblastoma,¹² tongue squamous cell carcinoma,¹⁵ laryngeal,¹⁷ breast,¹⁸ gastric,²⁰ prostate,¹³ ovarian,¹⁶ and cervical cancer.¹⁹ Nevertheless, miR-802 was demonstrated to function as an onco-miRNA in lung carcinoma¹⁴ and osteosarcoma.¹¹ Wang et al reported that miR-802 enhanced tumor cells proliferation via suppressing Menin in lung carcinoma;¹⁴ Cao et al showed that miR-802 could promote the proliferation of osteosarcoma cells through regulating p27.¹¹ However, the roles and potential mechanisms of miR-802 in the development and progression of CRC still remain largely unknown.

In the present study, we conducted a series of clinical sample detection, in vitro and in vivo experiments to explore the potential role and underlying mechanism of miR-802 in CRC development. The results found that miR-802 was significantly down-regulated in CRC tissues, and its low expression was significantly correlated with multiple unfavorable clinicopathological parameters and poor prognosis of CRC patients. Further in vitro and in vivo functional assays showed that miR-802 profoundly inhibited proliferation, migration and invasion but promoted apoptosis of CRC cells. Mechanistically, miR-802 functioned as a tumor suppressor through suppressing the expression of Ubiquitin-2 (UBN2) on post-transcriptional level in CRC. Taken together, these findings suggest that miR-802 plays a tumor suppressor role in CRC development, providing a promising biomarker and a potential treatment target for CRC.

Materials and Methods

Patient Samples

A total of 75 patients were recruited for the paired CRC and matched paracancerous tissues who undergone CRC surgery at Department of General Surgery, The First Affiliated Hospital of Jinzhou Medical University from January 2014 to December 2014. Inclusion criteria: 1) pathologically diagnosed as CRC; 2) with complete clinicopathological and follow-up data. Exclusion criteria: 1) accompanied by other malignant tumors, 2) infectious diseases, 3) receive anti-tumor treatment before surgery, 4) history of alcohol abuse or 5) mental illness. Samples were collected after tumor resection, one part stored in liquid nitrogen, and the another one embedded in paraffin. This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki, with authorization from Medical Ethics Committees of The First Affiliated Hospital of Jinzhou Medical University (No. 202026). Written informed consent was obtained before samples collection and analysis.

Cell Culture and Transfection

The human CRC cell lines (HCT116, HCT8, HT29 and DLD-1) and the human normal colonic mucosal cell line (NCM460) were originally obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 (Gibco, NY, USA) medium supplemented with 10% fetal bovine serum (FBS; HyClone, UT, USA) and incubated in a 5% CO₂, 37°C incubator.

MiR-802 mimics and control mimics, miR-802 inhibitor and control inhibitor were ordered from RiboBio (Guangzhou, China). The pcDNA-3.1-UBN2 plasmid was offered by GenePharma (Shanghai, China). Cell transfection was conducted with indicated plasmids and miRNAs when cells were grown to 60% confluence in 6-well plates by using Lipofectamine 2000 (Invitrogen, CA, USA) with RNA oligonucleotides at a final concentration of 50 nmol. In this study, control mimics and control inhibitor were transfected into CRC cells as a negative control. After transfection for 48 h, cells were collected for further experiments.

RNA Isolation and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) and purified using a miRNAeasy Kit (Qiagen, MD, USA). For mRNA, total RNA was reverse transcribed with a QuantiTect Reverse Transcription Kit

(Qiagen), and the double-stranded cDNA was amplified by using SYBR Premix Ex Taq (Takara, Dalian, China). For miRNA, total RNA was used for complementary DNA synthesis with a TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, MA, USA). qRT-PCR assays were carried out using a 7500 Real-Time PCR System (Applied Biosystems). U6 was used as a normalization control for miRNA, while GAPDH was used as a normalization control for mRNA. The relative mRNA expression level was calculated using the $2^{-\Delta\Delta C_t}$ method. PCR primers for miR-802 and U6 were designed and synthesized by RiBoBio. PCR primers for UBN2 and GAPDH were synthesized by GenePharma. The PCR primers were as following: miR-802 (Forward: CGTTGTG TAGCTTATCAGACTG, Reverse: AATGGTTGTTCTCC ACACTCTC); UBN2 (Forward: TTATATCAACACTGG CACTCTACA, Reverse: TTCCGCTTCCGCTTCTTC); U6 (Forward: CTCGCTTCGGCAGCACA, Reverse: AAC GCTTCAGGAATTTGCGT); GAPDH (Forward: GGAG CGAGATCCCTCCAAAAT, Reverse: GGCTGTTGTCAT ACTTCTCATGG).

Western Blot

Samples were lysed with RIPA lysis buffer (Beyotime, Shanghai, China) containing a protease inhibitor cocktail (Roche) for 20 min, and a BCA Protein Assay Kit (Beyotime) was used to determine the concentration. The primary antibodies including anti-human UBN2 antibody (1:1000 dilution; BD Biosciences, CA, USA) and anti-human GAPDH antibody (1:3000 dilution; Proteintech) were used.

Cell Proliferation and Apoptosis Assays

The proliferation of treated cells was analyzed with a Cell Counting Kit-8 (CCK-8; Beyotime) reagent according to the manufacturer's instructions. In brief, transfected CRC cells were placed into a 96-well plate with a density of 2×10^3 cells per well and cultured for 0, 24, 48, 72, 96 and 120 h. Then, 10 μ L/well of CCK8 reagent was added into each well. After incubation 2 h, the absorbance was measured at 450 nm using a microplate reader. The apoptosis assays were conducted with Annexin V-fluorescein isothiocyanate (FITC) detection kit (Sigma-Aldrich, Darmstadt, Germany). Briefly, after transfection for 48 h, cells were adhered to the wall and washed two times with pre-cooled $1 \times$ DPBS, and the cells were mixed up in $1 \times$ binding buffer (100 μ L). Then, Annexin V FITC PI detection kit was used for cell staining according to the

instructions. Finally, flow cytometer (BD Biosciences, USA) was used to analysis cells apoptosis.

Transwell Migration and Invasion Assays

The migration and invasion capabilities were evaluated by Transwell assays with an 8- μ m pore, 24-well Transwell plates (Corning, NY, USA). For cell migration assay, 2×10^5 transfected HCT116 or HT29 cells were added to the upper chamber, suspended in FBS-free RPMI 1640, while the lower chamber was filled with 750 μ L RPMI 1640 containing 10% FBS. For invasion assay, 2×10^5 of transfected cells were added into the upper chamber pre-coated with Matrigel (BD Biosciences). After incubation at 37°C with 5% CO₂ for 24 h, migrated or invaded cells on the lower membrane surface were stained with 0.1% crystal violet. Then, the stained cells were photographed and counted using a light microscope (Olympus, Tokyo, Japan).

Dual-Luciferase Reporter Gene Assay

A fragment of wild-type (WT) UBN2 3'-UTR (WT 3'-UTR) containing a miR-802 targeting site was amplified and cloned into psiCHECK™ vector (Promega, WI, USA). The mutant (MT) vector was constructed according to mutate the binding site region of miR-802. Then, HCT116 and HT29 cells were co-transfected with WT or Mut UBN2 3'-UTR reporter and miR-802 mimics or miR-802 inhibitor, respectively. Forty-eight-hour later, cells were lysed for luciferase assays, with the Dual Luciferase Assay (Promega) according to the manufacturer's instructions.

Immunohistochemistry

Serial sections at 4 μ m thickness were obtained from paraffin-embedded samples. Sections were incubated with rabbit anti-human UBN2 (1:100 dilution; BD Biosciences), mouse anti-human Ki-67 (1:200 dilution, Cell Signalling Technology, MA, USA) and mouse anti-human Cleaved Caspase-3 (1:200 dilution, Abcam, USA) overnight at 4°C after antigen retrieval and the block of non-specific binding. The sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (ZSGB, Beijing, China). Expression was visualized, and further independently scored based on the percentage of positively stained tumor cells by two observers.

Xenograft Experiments in Nude Mice

The 4–6 weeks old, 16–20 g in weight BALB/c female nude mice were randomly divided into two groups ($n=6$ per group), 5×10^6 HCT116 cells were subcutaneously injected into the hind limbs region of each mouse. After 8 days, miR agomir NC ($n=6$) or miR-802 agomir (RiboBio, Guangzhou, China) ($n=6$) was injected into the implanted tumor at the dose of 2 nmol/50 μ L PBS, respectively. After 30 days, mice were euthanized, and the weight and volume of tumors were further analyzed. Tumor volume was calculated as $1/2 \times (\text{width}^2 \times \text{length})$. Then, the xenograft tumors were collected for further experiments. All animal experiments were conducted with approval from the ethical committee of Animal Welfare Office of Jinzhou Medical University (No. 2020YK035), in accordance with the legal mandates and national guidelines for the care and maintenance of laboratory animals.

Statistical Analysis

All in vitro experiments were performed independently at least three times. Continuous data was presented as means $\pm$ standard deviations (SD), which was compared by using Student's *t*-test or analysis of variance, as appropriate. Categorical variables were represented as *n*, and Chi-square (χ^2) test was used for inter-group comparison. Correlation analysis was carried out by using Pearson's correlation analysis. Kaplan-Meier methods with Log rank test, univariate and multivariate Cox-regression analyses were used for prognostic analysis. All statistical analyses were conducted with SPSS 22.0 software (SPSS Inc., IL, USA), and $P < 0.05$ was considered representative of a significant difference.

Results

Decreased MiR-802 Expression Indicates Tumor Progression and Poor Prognosis in CRC

In order to investigate the role of miR-802 in the progression of CRC, qRT-PCR was firstly used to detect the expression levels of miR-802 in 75 pairs of CRC tissues and their matched paracancerous tissues. The results showed that decreased expression of miR-802 was observed in CRC tissues ($t=10.820$, $P < 0.001$; Figure 1A). In addition, results from patients with different tumor grade and stages also showed that down-regulation of miR-802 expression predicted poor tumor grade ($t=7.401$,

$P < 0.001$; Figure 1B) and advanced tumor stage ($t=6.612$, $P < 0.001$; Figure 1C).

Furthermore, we focused on the relationships between miR-802 expression in CRC tissues and patients' clinicopathological parameters. According to the median level of miR-802 expression, CRC patients were divided into high miR-802 expression group ($n=37$) and low miR-802 expression group ($n=38$). The results showed that low miR-802 expression was significantly correlated with poor tumor grade ($P=0.028$), late T stage ($P=0.039$), late N stage ($P=0.015$) and advanced TNM stage ($P=0.002$), but not with gender, age and tumor site ($P > 0.05$, respectively) (Table 1). Furthermore, Kaplan-Meier survival analysis demonstrated that patients with lower miR-802 expression exhibited significantly poorer overall survival (OS) rate ($\chi^2=5.746$, $P=0.017$; Figure 1D). Further univariate and multivariate Cox analyses confirmed that low miR-802 expression was an independent prognostic factor for affecting patient's OS (HR=1.964, 95% CI=1.116–3.460, $P=0.019$; Table 2) in CRC. Collectively, these results demonstrate that decreased miR-802 expression is associated with CRC progression and patient's poor prognosis, indicating miR-802 may act as a tumor-suppressor in CRC.

MiR-802 Suppresses Proliferation, Migration and Invasion and Promotes Apoptosis of CRC Cells in vitro

To further access the role of miR-802 in the development of CRC, we analyzed the effect of miR-802 on malignant behaviors of CRC cells in vitro. We firstly measured the expression level of miR-802 in four CRC cell lines and the human normal colonic mucosal cell line (NCM460). The results showed that miR-802 expression was significantly down-regulated in four CRC lines compared with NCM460 cells ($P < 0.05$, respectively; Figure 2A), in which, HCT116 presented the lowest miR-802 level while HT29 had the highest level. Thus, HCT116 and HT29 cells were selected for the gain- and loss-of-function assays with miR-802 mimics and miR-802 inhibitor, respectively. Further qRT-PCR results confirmed that miR-802 mimics significantly increased the expression of miR-802 in HCT116 cells ($P < 0.001$; Figure 2B), while miR-802 inhibitor significantly decreased the expression in HT29 cells ($P < 0.001$; Figure 2C).

Furthermore, we explored the effect of miR-802 on the proliferation and apoptosis of CRC cells via CCK-8 and Annexin V assay, respectively. The results showed that overexpression of miR-802 significantly inhibited

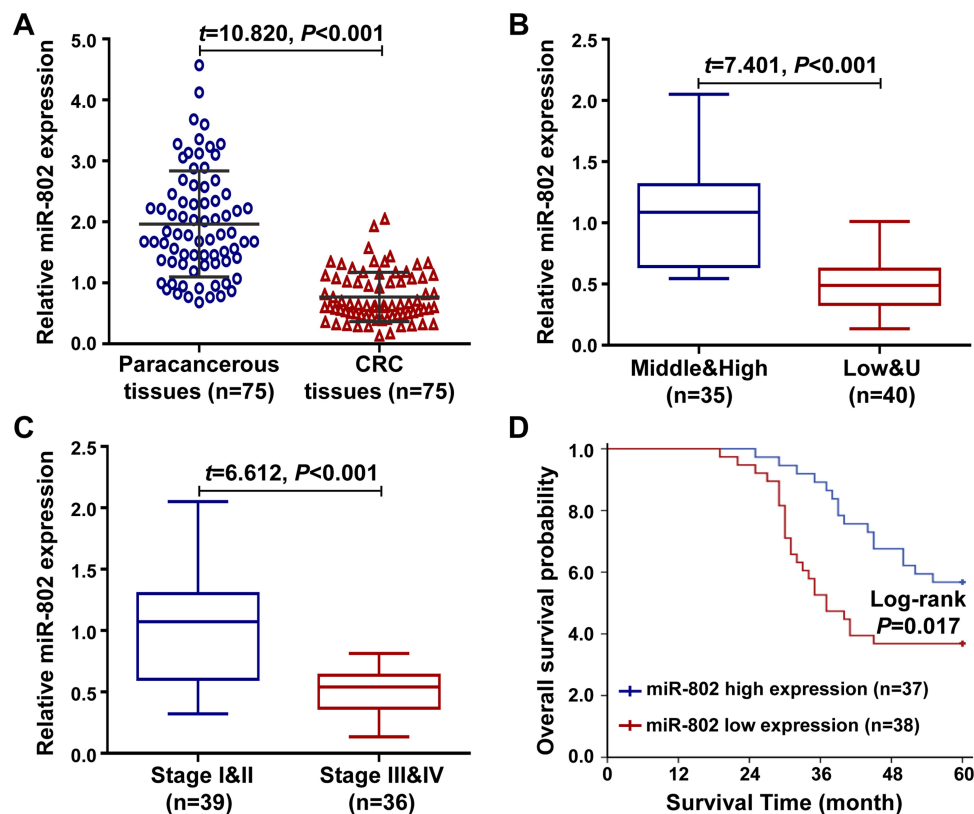


Figure 1 Decreased expression of miR-802 is related with CRC progression and poor prognosis. **(A)** MiR-802 expression in 75 of primary CRC tissues and paired paracancerous tissues detected by RT-PCR. **(B and C)** miR-802 expression in CRC tissue from patients with different tumor grades and clinical stages was analyzed by RT-PCR. **(D)** The Kaplan-Meier method was used to investigate the overall survival in CRC patients with different miR-802 expression levels.

Abbreviations: CRC, colorectal cancer; RT-PCR, quantitative real time-PCR.

HCT116 cells proliferation ability ($P<0.01$; Figure 2D), while increased their apoptosis rate ($P<0.01$; Figure 2E). In contrast, miR-802 knockdown significantly enhanced HT29 cells proliferation ability ($P<0.05$; Figure 2F), but decreased their apoptosis rate ($P<0.01$; Figure 2G). To further analyze the effect of miR-802 on tumor migration and invasion, migration and invasion assays of CRC cells infected with miR-802 mimics or inhibitor were performed. Upon miR-802 expression upregulation, the migration and invasion of CRC cells were markedly decreased ($P<0.01$, respectively; Figure 2H), while miR-802 knockdown increased the migratory and invasive abilities ($P<0.01$, respectively; Figure 2I). Taken together, these findings indicate that miR-802 suppresses the proliferation, migration and invasion but promotes the apoptosis of CRC cells in vitro.

UBN2 is Newly Identified as a Direct Target of MiR-802 in CRC Cells

Numerous studies have proved that miRNAs exert their functions mainly via regulating the expression of

downstream target genes.^{8–10} Thus, in order to explore the potential mechanisms of miR-802 on the malignant behaviors of CRC cells, three miRNA online databases including miRDB, miRWalk and TargetScan were used (Figure 3A). Among these predicated common targets, UBN2 was selected as the candidate target of miR-802 because it has been reported to promote the proliferation, migration and invasion of CRC cells in vitro and in vivo.²¹ We then detected the expression levels of UBN2 in a panel of CRC cell lines and NCM460 by qRT-PCR. The results showed that UBN2 expression was significantly upregulated in four CRC lines compared with NCM460 cells ($P<0.05$, respectively; Figure 3B). Furthermore, UBN2 expression was markedly upregulated in CRC tissues compared with paracancerous tissues ($P<0.001$; Figure 3C), and the expression of UBN2 mRNA was significantly negatively correlated with miR-802 level in CRC tissues ($r=-0.517, P<0.001$; Figure 3D).

Then, we assessed whether miR-802 directly binds to the 3'-UTR of *UBN2* mRNA in CRC cells. First, TargetScan prediction revealed that the 3'-UTR of *UBN2* mRNA contains a conserved binding site (2920~2926) for miR-802

Table 1 Correlations Between miR-802 Expression in CRC Tissues and Patients' Clinicopathological Parameters (n=75)

Parameter	Case	miR-802 Expression		χ^2 value	P value
		Low (n=38)	High (n=37)		
Gender				1.612	0.204
Male	44	25	19		
Female	31	13	18		
Age, years				3.127	0.077
≤60	32	20	12		
>60	43	18	25		
Tumor site				0.677	0.411
Colon	41	19	22		
Rectum	34	19	15		
Tumor grade				4.842	0.028*
Middle&High	39	15	24		
Low&U	36	23	13		
T stage				4.242	0.039*
T1&T2	24	8	16		
T3&T4	51	30	21		
N stage				5.945	0.015*
N0&N1	40	15	25		
N2&N3	35	23	12		
TNM stage				9.766	0.002*
I&II	39	13	26		
III	36	25	11		

Notes: * $P < 0.05$.

Abbreviations: CRC, colorectal cancer; U, undifferentiated; TNM, tumor-node-metastasis.

(Figure 3E). Subsequently, results from the dual-luciferase reporter assays showed that miR-802 overexpression markedly suppressed the luciferase activity of *UBN2* 3'-UTR

reporter wild-type construct ($P < 0.01$; Figure 3F), while miR-802 knockdown increased this activity ($P < 0.01$; Figure 3G). Whereas, the above effects were abolished by the introduction of a mutation into the miR-802-binding sequences in the mutant construct ($P > 0.05$, respectively; Figure 3F and G).

Moreover, Western blot and qRT-PCR were employed to evaluate the effects of miR-802 on *UBN2* protein and mRNA expression. The results showed that *UBN2* protein expression was significantly decreased in HCT116 cells transfected with miR-802 mimics, but significantly increased in HT29 cells transfected with miR-802 inhibitor (Figure 3H). Consistently, qRT-PCR results indicated that the expression of *UBN2* mRNA also exhibited a similar change trend of *UBN2* protein ($P < 0.01$, respectively; Figure 3I and J). Collectively, these findings indicate that miR-802 post-transcriptionally suppresses *UBN2* expression in CRC cells by directly targeting its 3'-UTR to degrade it.

Overexpression of *UBN2* Reverses the Biological Effects of CRC Cells Induced by MiR-802 Up-Regulation

To evaluate whether *UBN2* was required for the effect of miR-802 on CRC cell malignant behaviors, miR-802 mimics and *UBN2* plasmid were co-transfected into HCT116 cells. Further Western blot showed that of *UBN2* upregulation could reverse miR-802 overexpression-mediated *UBN2* protein repression (Figure 4A). Further functional experiments showed that overexpression of *UBN2* attenuated the inhibitive effects on cell proliferation, migration and invasion as well as the promotive effect on cell apoptosis mediated by miR-802 overexpression in HCT116 cells ($P < 0.01$, respectively; Figure 4B–D).

Table 2 Univariate and Multivariate Analyses of the Risk Factors for Overall Survival of CRC Patients

Parameter	Univariate analyses			Multivariate analyses		
	HR	95% CI	P value	HR	95% CI	P value
Gender (Female vs Male)	1.082	0.575–2.038	0.807	–	–	–
Age (≤60 vs >60 years)	1.463	0.771–2.776	0.245	–	–	–
Tumor site (Colon vs Rectum)	1.656	0.887–3.092	0.114	–	–	–
Tumor grade (Middle&High vs Low&U)	2.119	1.105–4.067	0.024*	1.722	0.880–3.371	0.113
T stage (T1&T2 vs T3&T4)	1.526	1.032–2.257	0.034*	1.367	0.645–2.842	0.116
N stage (N0&N1 vs N2&N3)	1.402	1.005–1.958	0.047*	1.146	0.526–1.895	0.203
TNM stage (I&II vs III&IV)	4.096	2.122–7.907	<0.001*	7.143	3.306–11.467	<0.001*
miR-802 expression (High vs Low)	2.119	1.122–4.002	0.021*	1.964	1.116–3.460	0.019*

Notes: –, Unavailable; * $P < 0.05$.

Abbreviations: CRC, colorectal cancer; U, undifferentiated; TNM, tumor-node-metastasis; HR, hazard ratio; CI, confidence interval.

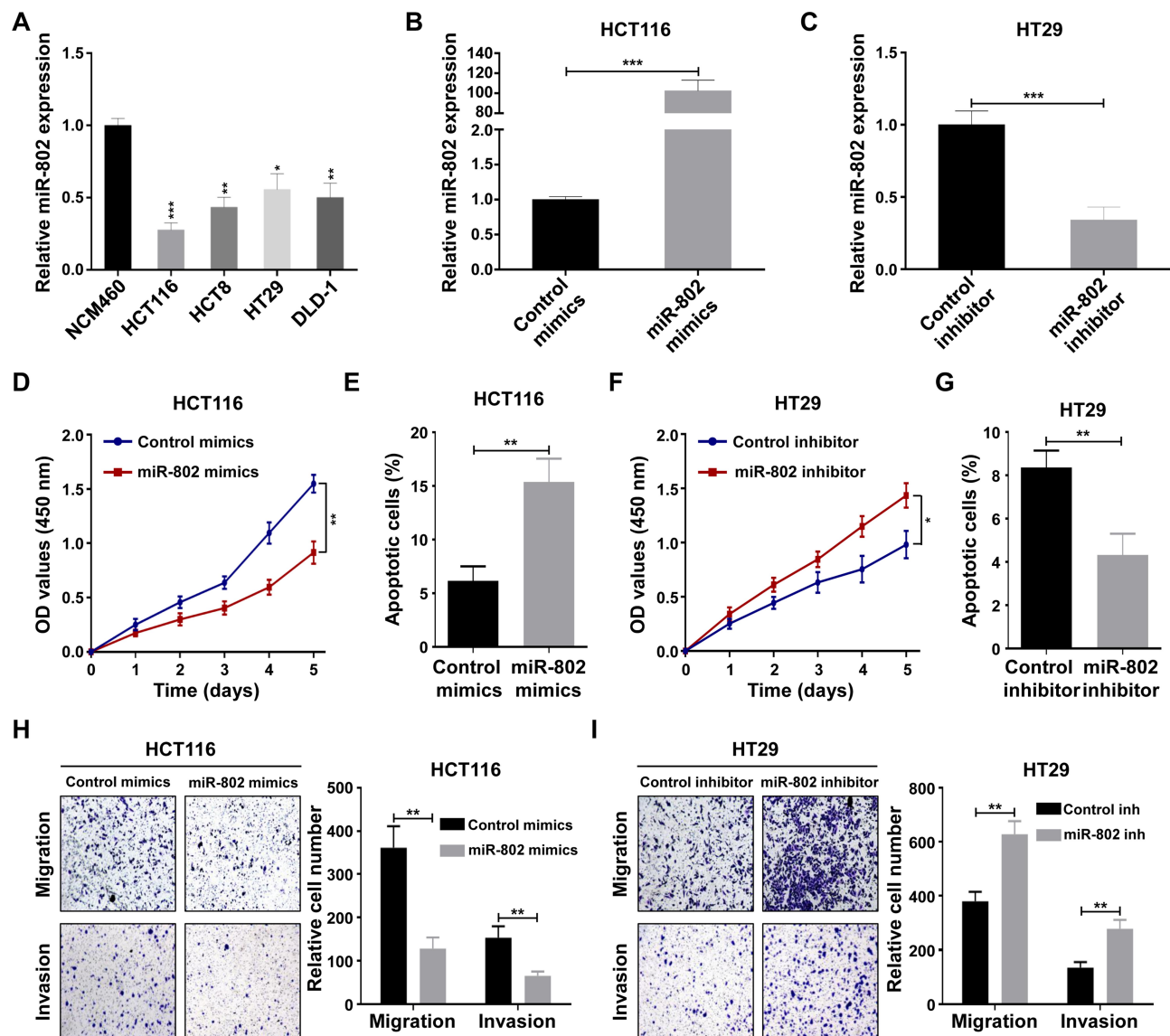


Figure 2 MiR-802 suppresses proliferation, migration and invasion and promotes apoptosis of CRC cells in vitro. (A) RT-PCR analysis of miR-802 expression in NCM460 and four CRC cell lines (HCT116, HCT8, HT29 and DLD-1). (B) RT-PCR analysis of miR-802 expression in HCT116 cells transfected with miR-802 mimics or control mimics at a final concentration of 50 nmol for 48 h. (C) RT-PCR analysis of miR-802 expression in HT29 cells transfected with miR-802 inhibitor or control inhibitor at a final concentration of 50 nmol for 48 h. (D and F) The proliferation capacity of miR-802-overexpressing HCT116 and miR-802-knockdown HT29 cells, as determined by CCK-8 assay. (E and G) Apoptosis rate of miR-802-overexpressing HCT116 and miR-802-knockdown HT29 cells, as determined by Annexin V assay. (H and I) The migration and invasion abilities of miR-802-overexpressing HCT116 and miR-802-knockdown HT29 cells.

Notes: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Abbreviations: CRC, colorectal cancer; RT-PCR, quantitative real time-PCR.

Together, these data suggest that miR-802 exerts its biological functions by suppressing UBN2 expression in CRC.

MiR-802 Suppresses Tumorigenesis of CRC Cells Through Targeting UBN2 in vivo

To explore the effects of miR-802 on tumorigenesis of CRC cells in vivo, we constructed nude mouse xenograft model via subcutaneously injecting HCT116 cells.

Subcutaneous tumorigenesis assay showed that miR-802 upregulation significantly inhibited tumor growth in nude mice (Figure 5A), and further analyses indicated the tumor weight ($P < 0.01$; Figure 5B) and tumor volume ($P < 0.01$; Figure 5C) were also significantly decreased in miR-802 agomir group, when compared with miR agomir NC group. Results about the expression of *UBN2* mRNA in tumor tissues showed that *UBN2* mRNA expression was significantly downregulated in tumors from miR-802

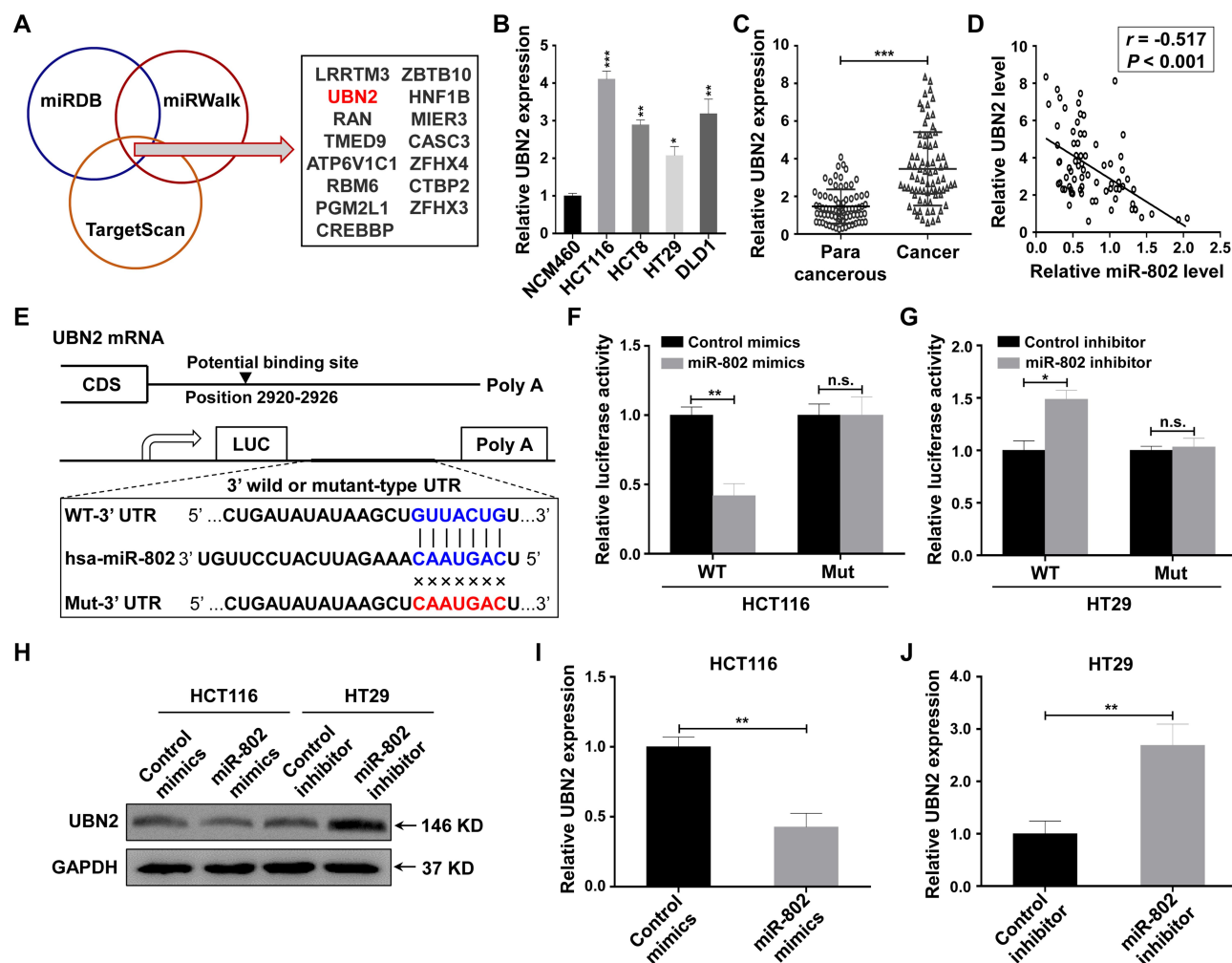


Figure 3 UBN2 is newly identified and validated as a direct target of miR-802 in CRC cells. (A) UBN2 was a potential target of miR-802 predicted by three miRNA online database, including miRDB, miRWalk and TargetScan. (B) RT-PCR analysis of UBN2 mRNA expression in NCM460 and four CRC cell lines (HCT116, HCT8, HT29 and DLD-1). (C) MiR-802 expression in 75 cases of primary CRC tissues and paired paracancerous tissues detected by RT-PCR. (D) Spearman's test analysis of the correlation between miR-802 and UBN2 mRNA expression in CRC tissues. (E) A schematic representation of the predicted duplex sequences between the WT or Mut 3'-UTR of UBN2 mRNA and miR-802. (F) Relative luciferase activity of UBN2 3'-UTR in HCT116 cells co-transfected with the indicated reporters and miR-802 mimics oligonucleotides. (G) Relative luciferase activity of UBN2 3'-UTR in HT29 cells co-transfected with the indicated reporters and miR-802 inhibitor oligonucleotides. (H) UBN2 protein expression in HCT116 cells transfected with miR-802 mimics and HT29 cells transfected with miR-802 inhibitor by Western blot. (I and J) RT-PCR analysis of UBN2 mRNA expression in HCT116 cells transfected with miR-802 mimics and HT29 cells transfected with miR-802 inhibitor.

Notes: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Abbreviations: CRC, colorectal cancer; UBN2, Ubinuclein-2; mRNA, messenger RNA; WT, wild-type; Mut, mutant; 3'-UTR, 3'-untranslated region; RT-PCR, quantitative real time-PCR.

agomir group ($P < 0.01$; Figure 5D). IHC staining revealed that miR-802 overexpression dramatically suppressed UBN2 protein expression ($P < 0.01$; Figure 5E) and Ki-67 index ($P < 0.001$; Figure 5F) in tumors. Moreover, the expression level of Cleaved Caspase-3 protein was significantly upregulated in tumors from miR-802 agomir group compared with miR agomir NC group ($P < 0.001$; Figure 5G). Taken together, these data indicate that miR-802 suppresses CRC tumorigenesis via targeting UBN2 to inhibit the proliferation but promote the apoptosis of CRC cells in vivo.

Discussion

The initiation and progression of cancer are a complex process controlled by multiple factors.²² Emerging evidence indicates that miRNAs act important roles in various malignant processes involved in CRC onset and development.²³ Moreover, numerous of miRNAs have been proposed as the novel prognostic markers for CRC.⁶ Thus, it is particularly necessary to further explore the biological roles and potential molecular mechanisms of miRNAs in the development of CRC. In this study, miR-802 was frequently down-regulated in CRC tissues

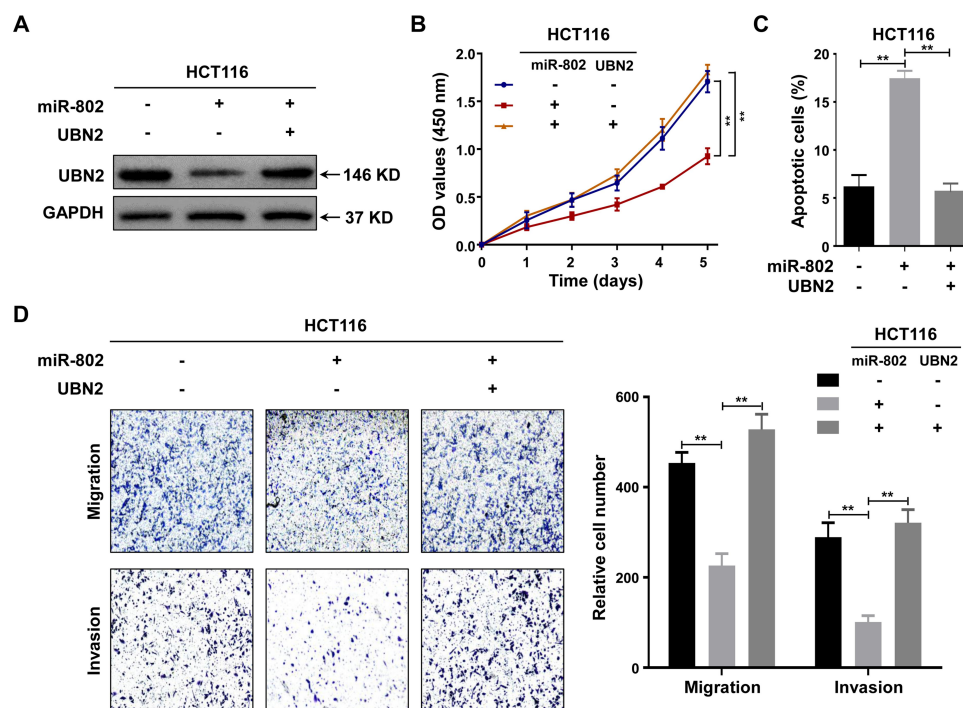


Figure 4 Overexpression of UBN2 reverses the biological effects of CRC cells induced by miR-802 up-regulation. HCT116 cells were transfected with UBN2 plasmid or a negative control, along with miR-802 mimics or corresponding control. **(A)** Western blot analysis of UBN2 protein expression in HCT116 cells. **(B)** The cell proliferation capacity of pretreated HCT116 cells, as determined by CCK-8 assay. **(C)** Apoptosis rate of pretreated HCT116 cells, as determined by Annexin V assay. **(D)** The migration and invasion abilities of pretreated HCT116 were analyzed by using Transwell assays or Matrigel-coated Transwell assays, respectively.

Notes: ** $P < 0.01$.

Abbreviations: CRC, colorectal cancer; UBN2, Ubinuclein-2; RT-PCR, quantitative real time-PCR.

and cells. Further analyses showed that miR-802 low expression in CRC tissues was significantly correlated with tumor progression and poor patients' prognosis. Further functional experiments showed that miR-802 could reduce CRC cells proliferation, migration and invasion while enhance the apoptosis of CRC cells in vitro and in vivo. Mechanistically, miR-802 exerted the above functions through inhibiting the expression of a cancer-promoting gene-UBN2 on post-transcriptional level. Collectively, these results indicate that miR-802 serves as a tumor suppressor in CRC, and might be proposed as a novel prognostic biomarker and a potential treatment target for CRC management. As far as we know, the present study firstly reveals the role and molecular mechanisms of miR-802/UBN2 axis in CRC development.

Numerous previous studies have indicated miR-802 was downregulation in multiple types of human cancers and played tumor suppressor role in cancer progression.^{12,13,15-20} Yuan et al firstly reported the inhibitory effect of miR-802 on cell proliferation via down-regulation of FoxM1 in breast cancer;¹⁸ Zhang and colleagues found that miR-802 was obviously down-

regulated in cervical cancer, which inhibited cell proliferation and induced apoptosis by targeting SRSF9;¹⁹ Wang et al found miR-802 inhibited epithelial-mesenchymal transition (EMT), migration and invasion of prostate cancer cells through targeting Flot2.¹³ In addition, recent studies also showed that miR-802 acted as a tumor suppressor in gastric cancer, non-small cell lung cancer, epithelial ovarian cancer, laryngeal cancer, tongue squamous cell carcinoma and glioblastoma via, respectively, suppressing down-stream multiple oncogenes expression, including RAB23, FGFR1, YWHAZ, ARPP19, MAP2K4 or SIX4.^{12,15-17,20,24} Herein, our clinical results showed that miR-802 was significantly down-regulated in CRC tissues, and lower miR-802 expression was apparently associated with poorer tumor grade, later T stage, later N stage and advanced TNM stage and worse patient's OS. Furthermore, elevated expression of miR-802 suppressed CRC cells proliferation, migration and invasion while enhanced the apoptosis of CRC cells by directly targeting UBN2 both in vitro and in vivo. Our results were consistent with the recent published study conducted by Wang et al, which showed that the low expression of miR-802 in CRC tissues was related to shorter patient's

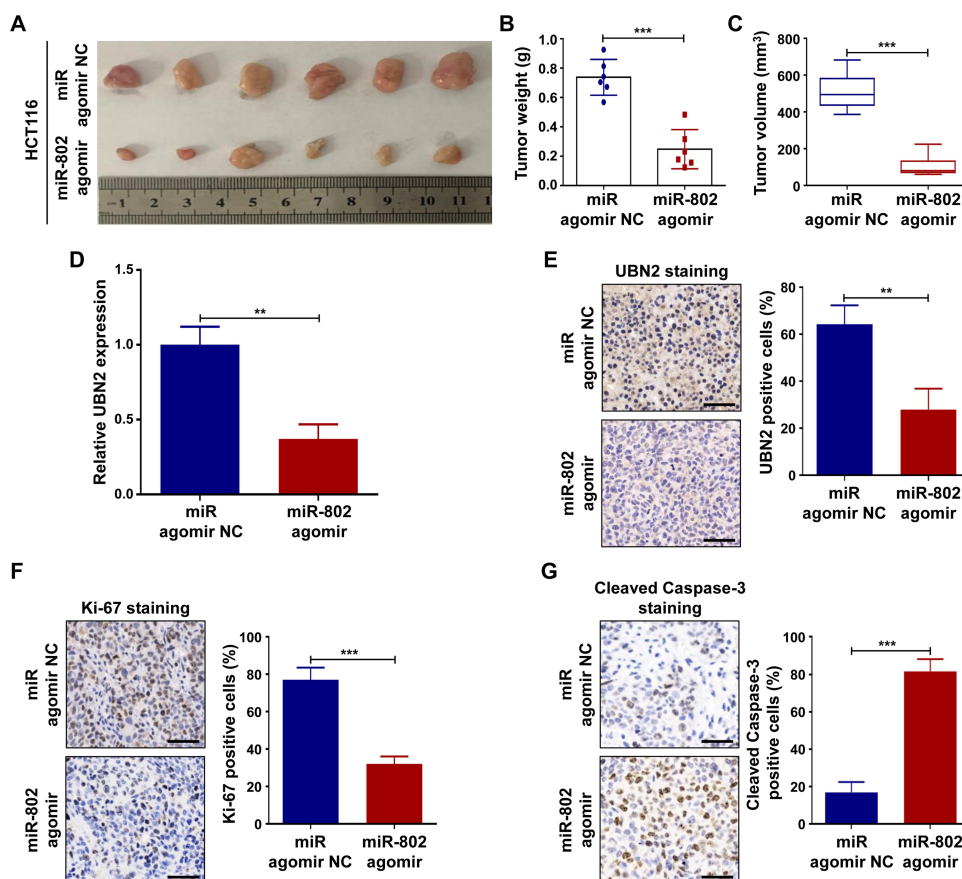


Figure 5 MiR-802 suppresses tumorigenesis of CRC cells through targeting UBN2 in vivo. **(A)** The morphological characteristics of tumor xenograft of HCT116+miR agomir NC and HCT116+miR-802 agomir groups. **(B and C)** Tumor weight and volume of xenograft in HCT116+miR agomir NC and HCT116+miR-802 agomir groups. **(D)** RT-PCR analysis of UBN2 mRNA expression in tumor from HCT116+miR agomir NC and HCT116+miR-802 agomir groups. **(E–G)** IHC detection of UBN2, Ki-67, and Cleaved Caspase-3 protein expression in tumor from HCT116+miR agomir NC and HCT116+miR-802 agomir groups, respectively. Scale bars, 50 μ m.

Notes: ** $P < 0.01$; *** $P < 0.001$.

Abbreviations: CRC, colorectal cancer; UBN2, Ubinuclein-2; mRNA, messenger RNA; RT-PCR, quantitative real time-PCR.

OS.²⁵ Together, these data suggest that miR-802 acts as tumor suppressor in CRC.

The UBN2 protein is encoded by the *UBN2* gene that is located on chromosome 7q34,²⁶ which belongs to the ubinuclein family and widely exists in human adult and fetal tissues.²⁷ Previous study found that UBN2 was widely expressed in tumor tissues.²⁸ *UBN1*, as an important paralog gene of *UBN2*, was reported to be abnormally expressed in various cancers, including leukemia, lung carcinoma, cervical cancer, melanoma, pancreatic adenocarcinoma, as well as CRC.²⁹ Recently, Zhao et al found that UBN2 was highly expressed in CRC tissues through bioinformatics analysis and clinical sample detection, and high UBN2 expression was associated with tumor progression and poor outcome in CRC patients. Further in vitro and in vivo experiments revealed that UBN2 promoted CRC cells proliferation through Ras/MAPK pathway.²¹ These findings demonstrate the tumor-promoting effect

of UBN2 in CRC. In this study, *UBN2* was newly identified and validated as a direct target gene of miR-802 in CRC cells by using luciferase reporter assay, qRT-PCR and Western blot. Moreover, the UBN2 expression in CRC tissues was significant upregulated, and was inversely associated with the expression of miR-802. Further rescue experiments demonstrated that overexpression of UBN2 could reverse miR-802 overexpression-induced the repression of CRC cells proliferation, migration and invasion. These data suggest that miR-802 plays tumor-suppressive role in CRC partially via inhibiting UBN2 expression.

Conclusions

In summary, our study shows that the expression of miR-802 is downregulated in CRC tissues, and its low expression is significantly associated with poor prognosis of CRC patients. Further exploration demonstrates that

miR-802 inhibits the proliferation, migration and invasion while promotes the apoptosis of CRC cells via directly suppressing UBN2 expression. These findings highlight the important role of miR-802/UBN2 axis in suppressing CRC progression, providing promising biomarkers and potential treatment targets for CRC.

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Disclosure

The authors declare that they have no competing interests in this work.

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