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Circ_0001982 accelerates the progression of colorectal cancer *via* sponging microRNA-144

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Abstract. – OBJECTIVE: The aim of this study was to uncover the expression pattern and biological function of circ_0001982 in the progression of colorectal cancer (CRC).

PATIENTS AND METHODS: Relative expression level of circ_0001982 in 66 paired CRC tissues and adjacent normal tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The association between circ_000[illegible]82 level and clinical indexes of CRC patients [illegible]sessed. The effect of circ_0001982 on cellu[illegible]e-haviors of HT29 and HCT-116 cells was eval[illegible]d *in vitro*. Dual-Luciferase reporter gene assay conducted to verify the binding relation betwe[illegible] circ_0001982 and microRNA-14[illegible]y, rescu[illegible] experiments were performed [illegible]se[illegible]e role o[illegible] the circ_0001982/microRNA [illegible]axis i[illegible]ediating the progression of CRC.

RESULTS: Circ_0001982 v[illegible]anif[illegible]ulated in CRC tissues [illegible]n co[illegible]with adjacent normal ones. CRC [illegible]nts with [illegible] expression level of circ_000[illegible]howed a sig[illegible]ntly higher rate of dist[illegible]sis and wo[illegible]survival. Knockdown o[illegible]c_000[illegible]emarkably attenuated the prolife[illegible]e, migratory [illegible]invasive capacities of HCT-1[illegible]ells. However, [illegible]ite results were obser[illegible]fter th[illegible]overexpress[illegible]n of circ_0001982 in H[illegible]cells. M[illegible]RNA-144 was verified as a target ge[illegible]circ[illegible]1982, which could be negatively regula[illegible]irc_000[illegible]. Furthermore, microR-[illegible]144 w[illegible]pable[illegible]eversing the regulatory ef-[illegible]f circ_[illegible]n the proliferative, migratory, an[illegible]asive c[illegible]es of CRC cells.

C[illegible]NCLUSIONS: Up-regulated circ_0001982 w[illegible]lated to distant metastasis and [illegible]r prog[illegible]is of CRC. In addition, circ_0001982 [illegible]tenuated the progression of CRC by negative-[illegible]gulating microRNA-144.

Key Words:
Circ_0001982, MiRNA-144, Colorectal cancer (CRC), Malignant progression.

Introduction

[illegible]orectal cancer (CR[illegible]) ranks third and [illegible]th in morbidi[illegible] and mortality globally, re-[illegible]tively[1,2]. Su[illegible]al procedures accompanied b[illegible]stoperativ[illegible]emotherapy or radiotherapy are [illegible]red[illegible]gies for CRC[3,4]. Nevertheless, postope[illegible]ecurrence and metastasis often lead to poor prognosis of CRC patients[5,6]. Cur-[illegible]the choice of postoperative therapeutic [illegible]s is based on the TNM staging of CRC patients[6]. However, there is still a lack of biological hallmarks for predicting tumor recurrence and metastasis. Therefore, it is significant to develop prognostic hallmarks for early-stage intervention and improvement of overall survival of CRC[1,7,8].

As a classical RNA molecule, circRNA forms a covalently closed continuous loop. It was first discovered in viroids in 1976[9,10]. CircRNA is widely involved in intervening gene expression and regulation. Functionally, circRNA regulates the transcription of target mRNAs by sponging miRNAs. CircRNA can also form protein complexes accompanied by RNAs. A small part of circRNAs is able to synthesize proteins as transcriptional templates[11,12]. CircRNA has been found widely distributed in cell components, showing time-, tissue- and disease-specificity[12]. Due to the lack of 3' and 5' ends structure, circRNA is resistant to exonuclease. Furthermore, stably expressed circRNA in exosomes, cells, or tissues makes it a promising hallmark for tumor diagnosis and prognosis[13-15].

In this paper, we first analyzed the expression of circ_0001982 in CRC and investigated its function *in vitro*. After predicting and verifying the target gene of circ_0001982, we further explored their interaction in the progression of CRC.

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Patients and Methods

Patients and CRC Samples

Paired CRC tissues and adjacent normal tissues were surgically resected from 66 CRC patients. None of the patients received preoperative anti-tumor therapies. Clinical indexes were collected from CRC patients for further analyses. Informed consent was obtained from patients and their families before the investigation. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine.

Cell Lines and Reagents

CRC cell lines (HT29, HCT8, and HCT-116) and colorectal mucosal cell line (FHC) were provided by ATCC (Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. A culture medium was replaced every 2-3 days. The cell passage was conducted at 80-90% of confluence.

Cell Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). Cells were first pre-seeded into 6-well plates. Cell transfection was performed according to the instructions of Lipofectamine 2000 at 70% of confluence. After 48 h, transfected cells were collected for following experiments.

Cell Proliferation Assay

Cells were seeded in 96-well plates at a density of 2× cells per well at established time points. Absorbance (A) at 450 nm of each sample was detected using Cell Counting Kit (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Finally, viability curve of cells was plotted.

Transwell Invasion Assay

Transfected cells for 48 h were adjusted to a density of 2.0×10^5/mL. Briefly, 200 µL/well cell suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA) pre-coated with Matrigel. Meanwhile, 700 µL medium containing 10% FBS was added to the lower side. After 48 h of incubation, invasive cells to the bottom side were fixed with methanol for 15 min and stained with crystal violet for 20 min. Penetrating cells were observed under a microscope and the number of invading cells was counted. 5 fields of view were randomly selected for each sample.

Wound Healing Assay

Cells were seeded into 6-well plates at a density of 5.0×10^5 cells/well. Until 90% confluence, 1 mL pipette tip was used for creating artificial wound in the confluent cell monolayer. The percentage of wound closure was calculated at 0 and 24 h, respectively.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified by DNase I treatment. Extracted RNA was reversely transcribed into cDNA using PrimeScript RT Reagent (Takara, Otsu, Shiga, Japan). Obtained cDNA was subjected to qRT-PCR using SYBR® Premix Ex Taq™ (Takara, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal reference for mRNA and miRNA, respectively. Each sample was performed in triplicate. The relative expression level of the gene was calculated by $2^{-\Delta\Delta Ct}$ method and analyzed by iQ5 2.0. Primers used in this study were as follows: Circ_0001982: forward: 5'-TAGCAGTTCCCCAATCCTTG-3', reverse: 5'-CACAAATTCCCATCATTCCC-3'; GAPDH: forward: 5'-CGCTCTCTGCTCCTC CTGTTC-3', reverse: 5'-ATCCGTTGACTC-CGA CCTTCAC-3'; miRNA-144: forward: 5'-GGAGAAACGCCG CCACGTATCC-3', reverse: 5'-GCTCGATGGG AGCGATGGACC-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Dual-Luciferase Reporter Gene Assay

Cells were co-transfected with pmirGLO-circ_0001982-WT/pmirGLO-circ_0001982-MUT/pmirGLO and microRNA-144 mimics/NC using Lipofectamine 2000. 24 h later, co-transfected cells were harvested. The luciferase activity was determined in accordance with the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (Version X; La Jolla, CA, USA) was used for all statistical analyses. Experimental data were expressed as mean ±

standard deviation (SD). Intergroup differences were analyzed by the *t*-test. Kaplan-Meier curve was introduced to assess the prognosis of CRC patients. Spearman regression test was performed to evaluate the relation between the two genes. $p < 0.05$ was considered statistically significant.

Results

Circ_0001982 Was Highly Expressed in CRC Tissues and Cells

A total of 66 paired CRC tissues and adjacent normal tissues were collected. Circ_0001982 was found significantly up-regulated in CRC tissues when compared with normal tissues (Figure 1A, 1B). Identically, circ_0001982 was significantly up-regulated in CRC cell lines compared to the colorectal mucosal cell line (Figure 1C). Results suggested that circ_0001982 might exert a carcinogenic role in the progression of CRC.

Circ_0001982 Expression Was Correlated with Distance Metastasis and Overall Survival of CRC Patients

The correlation between the circ_0001982 expression level and clinical indexe[s] patients was assessed. As shown [in] Table 1, circ_0001982 level was positively [re]lated with distant metastasis, whereas it was [un]rrelated with age, gender, tumor stag[e] and lym[ph] me-tastasis of CRC patients. T[he] Kaplan-Mei[er] was depicted by analyz[ing] the foll[ow]-up da[ta] CRC patients. The re[sult] indica[ted] that the prog-nosis of CRC pati[ents] [with] expre[ssion] of circ_0001982 w[as] mark[edly] worse (Figure 1D).

Knockdo[wn] [of] circ_0001[982] Suppressed the Prol[ifer]ation, Migration, and Invasion of C[RC] Cells

W[e] constructed pcD[NA]-circ_0001982 and an-ti-circ_0001982 for altering the circ_0001982 ex-pression. Transfection of pcDNA-circ_0001982 significantly up-regulated circ_0001982 lev-

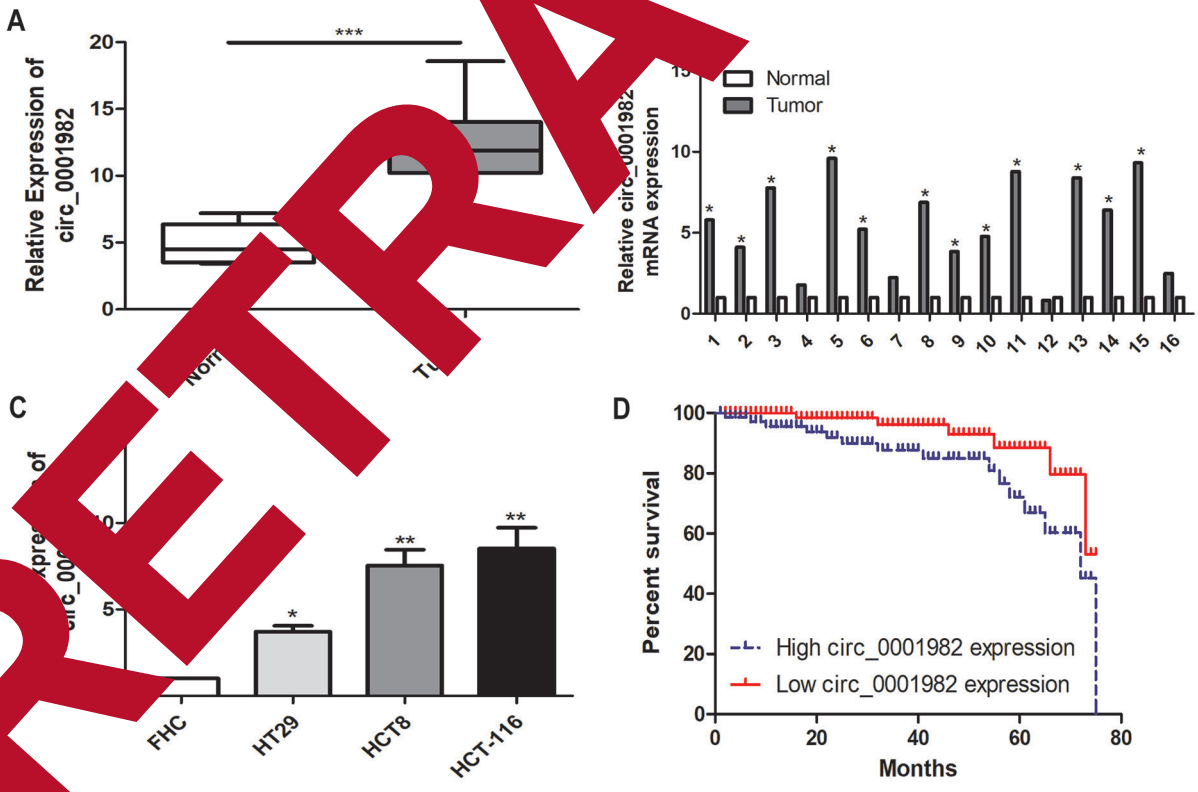

Figure 1. Circ_0001982 was highly expressed in CRC tissues and cell lines. **A,** Relative level of circ_0001982 in CRC tissues and adjacent normal tissues (n=66). **B,** Relative level of circ_0001982 in16 paired CRC tissues and adjacent normal tissues. **C,** Relative level of circ_0001982 in CRC cell lines (HT29, HCT8 and HCT-116) and colorectal mucosal cell line (FHC). **D,** Kaplan-Meier curves revealed the overall survival of CRC patients with high and low expression of circ_0001982.

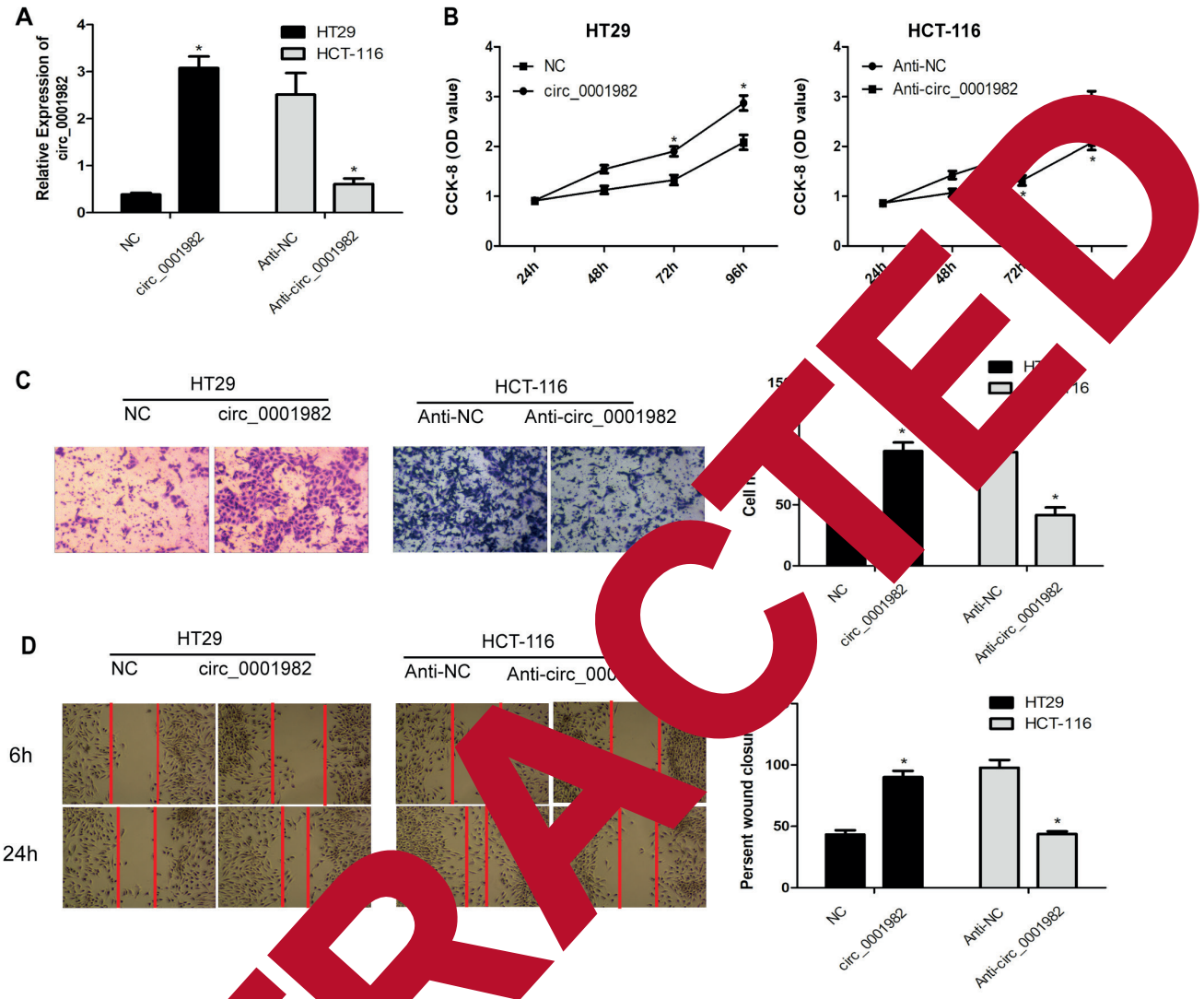

Figure 2. Knockdown [illegible]c_0001982 [illegible]ressed the proliferation, migration and invasion of CRC cells. **A,** Transfection efficacy of pcDNA-circ [illegible]82 in HT29 ce[illegible]anti-circ_0001982 in HCT-116 cells. **B,** CCK-8 assay showed viability of HT29 cells transfected w[illegible]NA-circ_000[illegible]d HCT-116 cells transfected with anti-NC/anti-circ_0001982. **C,** Transwell assay showed in[illegible]n of H[illegible]lls transfected with NC/pcDNA-circ_0001982 and HCT-116 cells transfected with anti-NC/anti-circ_000198[illegible] (magnificatio[illegible] **D,** Wound healing assay showed the percentage of wound closure in HT29 cells transfected with NC/p[illegible]-circ_0001982 a[illegible]T-116 cells transfected with anti-NC/anti-circ_0001982 (magnification × 40).

[illegible]n HT[illegible]lls. H[illegible]ever, transfection of anti-[illegible]_000[illegible]ificantly down-regulated its lev[illegible]n HCT-[illegible]cells (Figure 2A). A series of fu[illegible]experiments revealed that the overexpressio[illegible]rc_0001982 in HT29 cells significantly accelerated cell viability, the number of invasive cells, and the percentage of wound closure (Figure 2B-2D, left). On the contrary, opposite results were observed after the transfection of anti-circ_0001982 in HCT-116 cells (Figure 2B-2D, right).

MicroRNA-144 Was Down-regulated in CRC

MicroRNA-144 was predicted as the potential target of circ_0001982 in CRC through a bioinformatics method (data not shown). Compared with adjacent normal tissues, microRNA-144 was significantly down-regulated in CRC tissues (Figure 3A). Meanwhile, microRNA-144 was lowly expressed in CRC cell lines as well (Figure 3B). 16 CRC tissues were selected and the relation between circ_0001982 and microRNA-144

Table I. Association of circ_0001982 expression with clinicopathologic characteristics of colorectal cancer.

Parameters	No. of cases	Circ_0001982 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.[illegible]
<60	28	16	12	
≥60	38	17	21	
Gender				0.4[illegible]
Male	33	18	15	
Female	33	15	18	
T stage				0.035
T1-T2	38	22	16	
T3-T4	28	11	17	
Lymph node metastasis				0.205
No	41	23	18	
Yes	25	10	15	
Distance metastasis				[illegible]
No	38	23	[illegible]	
Yes	28	10	[illegible]8	

was evaluated. Spearman regression test showed that a negative relation was observed between circ_0001982 expression and microRNA-14[illegible] pression in CRC tissues (Figure 3C). By [illegible] ing the follow-up data, CRC patients with [illegible] w level of microRNA-144 presented significa[illegible] worse survival (Figure 3D).

To further explore the int[illegible] betwe[illegible] circ_0001982 and microRNA-1[illegible] Lucifer- ase reporter gene assay was [illegible]ucted. [illegible] rexpression of microRNA-144 r[illegible]ably [illegible] luciferase activity of w[illegible]-ty[illegible]01982, [illegible]- gesting the binding [illegible]ion bet[illegible]circ_0001982 and microRNA-14[illegible]ure 3E).

Circ_0001[illegible] Mod[illegible]d CRC Progres[illegible] by Negati[illegible] Regulating Micro[illegible]-144 Expressio[illegible]

M[illegible]RNA-1[illegible] has been proved to interact wi[illegible]_00[illegible]2. Nex[illegible] we speculated whether circ_[illegible] sponge[illegible]croRNA-144 to medi- cellu[illegible] havi[illegible]f CRC cells. First of all, t[illegible]nsfec[illegible]acy of microRNA-144 mim- ics [illegible] inhibit[illegible] HT29 and HCT-116 cells was ve[illegible] qRT-PCR (Figure 4A). Transfection [illegible]-144 mimics in HT29 cells overex- pressing circ_0001982 markedly increased cell [illegible]ility, the percentage of wound closure, and th[illegible]mber of invasive cells (Figure 4B-4D). Conversely, transfection of microRNA-144 inhibitor in HCT-116 cells with circ_0001982 knockdown yielded the opposite results (Figure 4B-4D).

[illegible]iscussion

CRC [illegible]inal tumor, whose prognosis de- pends on TNM staging at the first time of diagnosis. Unlike other malignancies, high-risk adenomas [illegible]arly-stage CRC are possible to be cured[4]. The postoperative 5-year survival of early-stage CRC is up to 90%[5,6]. Current reports have found that the etiology and pathogenesis of CRC are extremely complex. Epigenetic changes and gene mutations are the major reasons for the tumorigenesis of CRC. Meanwhile, genetic factors can also result in the occurrence of CRC (i.e., Lynch syndrome)[7]. In recent years, several researches have mainly focused on developing the effective hallmarks for screening and diagnosing CRC.

Abundant circRNAs have been discovered by bioinformatics methods and sequencing technologies[9-11]. Meanwhile, vital functions of circRNAs in the occurrence and progression of tumors have been extensively concerned, especially in the digestive system tumors[16]. CircRNAs can sponge corresponding miRNAs and block their functions, thereby altering the expressions of downstream miRNAs[14,15]. In this study, we revealed that circ_0001982 was significantly up-regulated in CRC. Meanwhile, circ_0001982 expression was related to distant metastasis and overall survival of CRC patients. Overexpression of circ_0001982 remarkably promoted the proliferative, migratory, and invasive capacities of CRC cells. Conversely, opposite results were observed after the knock-

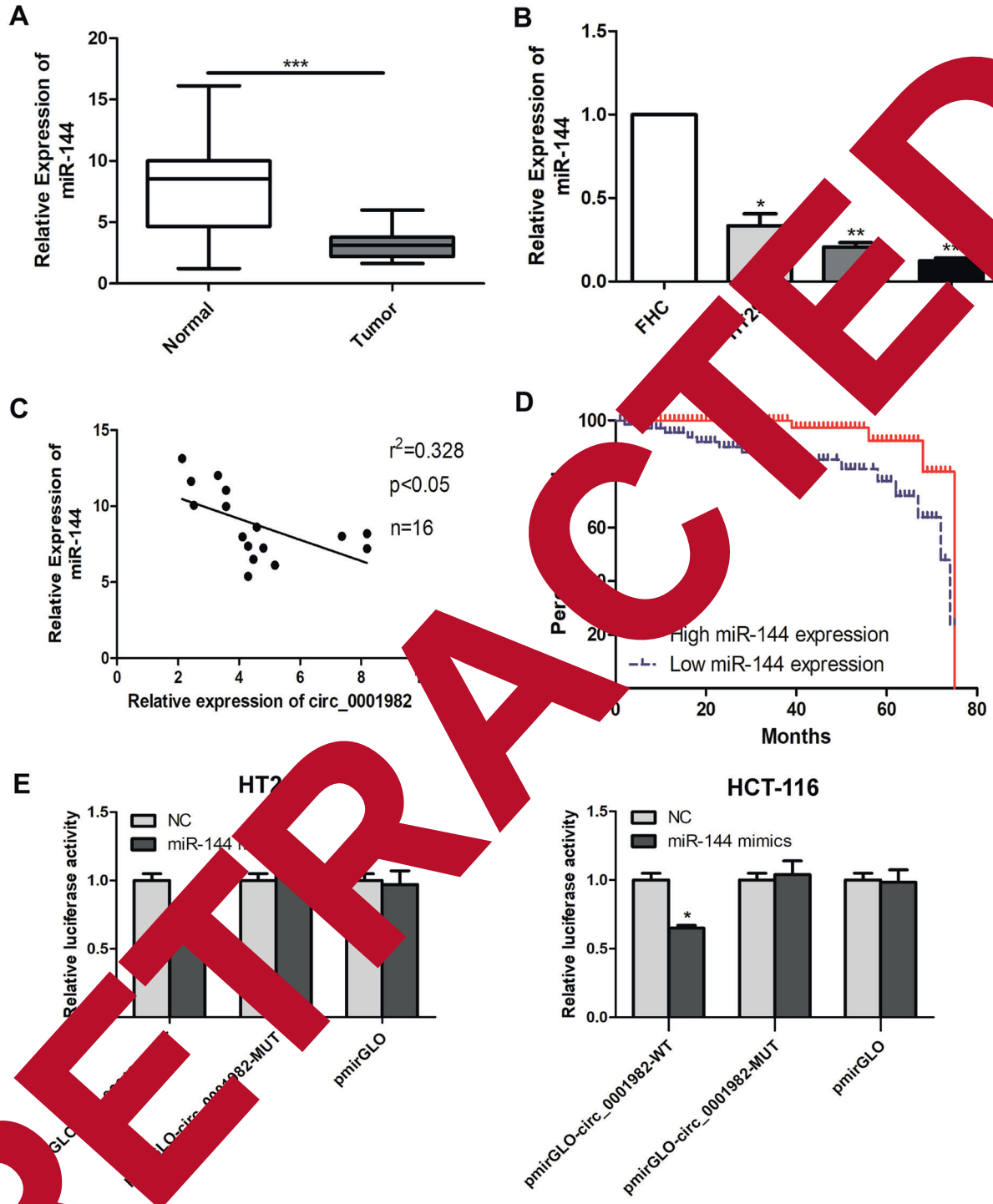

Figure 3. MiR-144 was down-regulated in CRC. **A,** Relative level of miR-144 in CRC tissues and adjacent normal tissues (n=66). **B,** Relative level of miR-144 in CRC cell lines (HT29, HCT8 and HCT-116) and colorectal mucosal cell line (FHC). **C,** Negative correlation between expression levels of circ_0001982 and miR-144 in CRC tissues. **D,** Kaplan-Meier curves revealed the overall survival of CRC patients with high and low expression of miR-144. **E,** Dual-Luciferase reporter gene assay showed relative luciferase activity in HT29 and HCT-116 cells co-transfected with pmirGLO-circ_0001982-WT/pmirGLO-circ_0001982-MUT/pmirGLO and miR-144 mimics/NC.

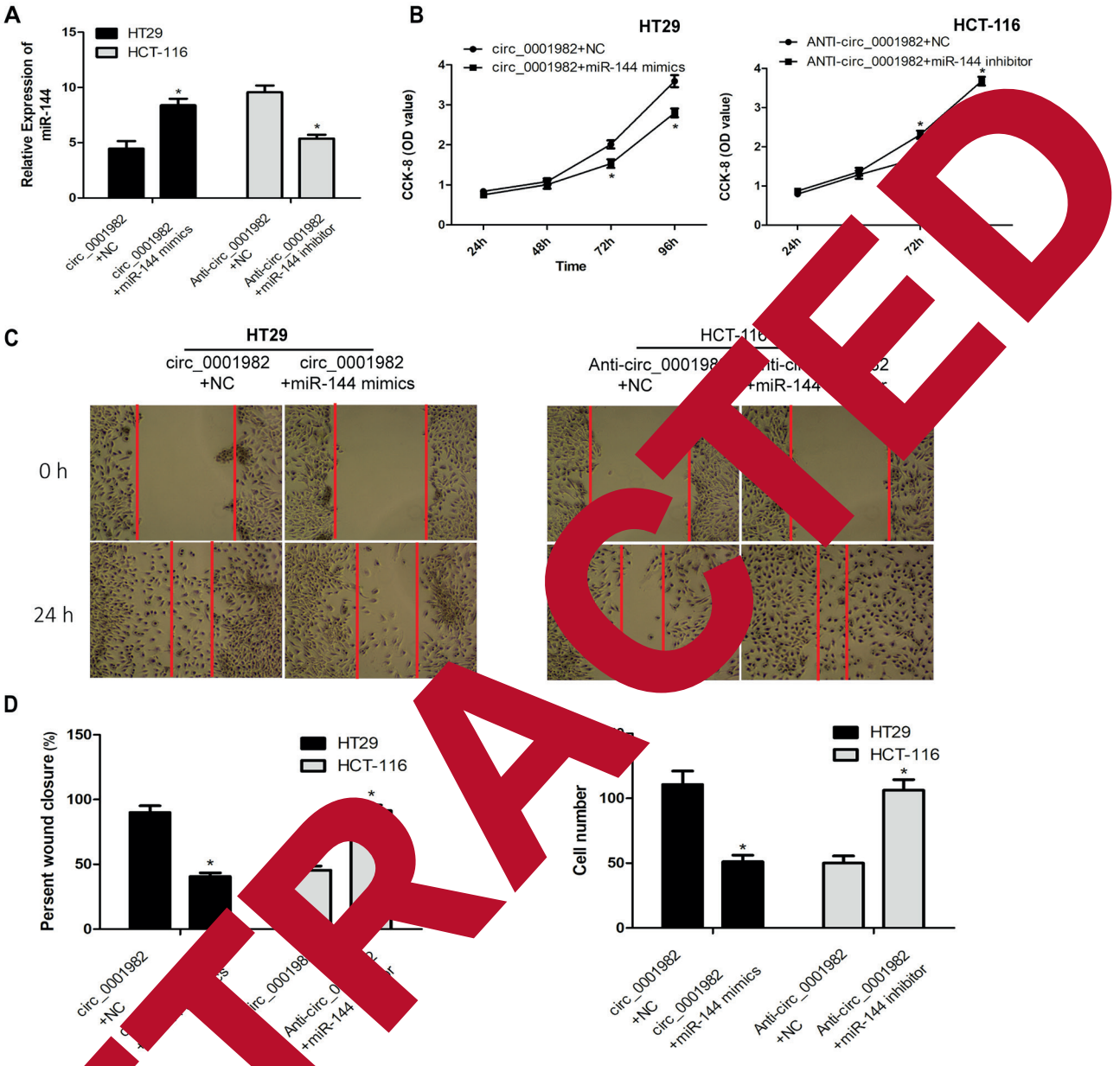

Figure circ_0001982 modulates CRC progression by negatively regulating miR-144 expression. **A,** Relative level of miR-144 in HT29 cells transfected with pcDNA-circ_0001982+NC/pcDNA-circ_0001982+miR-144 mimics and HCT-116 cells transfected with anti-circ_0001982+NC/anti-circ_0001982+miR-144 inhibitor. **B,** CCK-8 assay showed the viability of HT29 cells transfected with pcDNA-circ_0001982+NC/pcDNA-circ_0001982+miR-144 mimics and HCT-116 cells transfected with circ_0001982+NC/anti-circ_0001982+miR-144 inhibitor. **C,** Wound healing assay showed the percentage of wound closure in HT29 cells transfected with pcDNA-circ_0001982+NC/pcDNA-circ_0001982+miR-144 mimics and HCT-116 cells transfected with anti-circ_0001982+NC/anti-circ_0001982+miR-144 inhibitor (magnification × 40). **D,** Transwell assay showed the migration of HT29 cells transfected with pcDNA-circ_0001982+NC/pcDNA-circ_0001982+miR-144 mimics and HCT-116 cells transfected with anti-circ_0001982+NC/anti-circ_0001982+miR-144 inhibitor.

n of circ_0001982 *in vitro*. The above results suggested that circ_0001982 exerted a carcinogenic role in CRC.

Furthermore, we predicted the miRNA sponged by circ_0001982 through the bioinformatics method. Finally, microRNA-144 was screened out. QRT-PCR results demonstrated that microRNA-144 was lowly expressed in CRC tissues and cell lines. CRC patients with low expression of microRNA-144 presented a significantly worse survival relative to

those with a high level. These findings verified that circ_0001982 could directly bind to microRNA-144 in CRC cells. Notably, microRNA-144 was capable of reversing the regulatory effect of circ_0001982 on the proliferative, migratory, and invasive abilities of CRC cells. Therefore, circ_0001982 accelerated the malignant progression of CRC *via* negatively regulating microRNA-144.

Conclusions

Collectively, up-regulated circ_0001982 was closely related to distant metastasis and poor prognosis of CRC. Furthermore, circ_0001982 could attenuate the progression of CRC by negatively regulating microRNA-144.

Conflict of Interest

The Authors declare that they have no conflict of interests

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