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MiR-30c exerts tumor suppressive functions in colorectal carcinoma by directly targeting BCL9

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Abstract. – OBJECTIVE: Colorectal carcinoma (CRC) remains a leading health threat worldwide due to its high mortality. MicroRNA (miR-30c) is an important tumor suppressor in various cancers. B cell lymphoma 9 (BCL9) is one of the candidate genes for cancers. The synergistic effects of miR-30c and BCL9 in CRC progression remain to be carefully elucidated.

PATIENTS AND METHODS: Fifty pairs of CRC samples and matched adjacent non-tumor tissues were collected from Yantai Yuhuangding Hospital between 2015 and 2017. MiR-30c and BCL9 expression levels were measured by quantitative Real-time polymerase chain reaction (qRT-PCR) in CRC tissues and cell lines. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the influence of miR-30c on the proliferation ability of CRC cells. Target Scan was used to predict the potential target of miR-30c. Then, luciferase assay was performed to confirm the prediction. In addition, we also investigated the biological influence of BCL9 on miR-30c-mediated functions in CRC.

RESULTS: We found that miR-30c was significantly decreased in CRC tissues and cell lines while the BCL9 expression level was prominently increased in CRC tissues and cells. Additionally, the miR-30c expression was negatively correlated with BCL9 expressions in CRC tissues. Furthermore, the findings of this study also showed that BCL9 was a direct target of miR-30c in CRC and miR-30c could inhibit the CRC proliferation by binding to its 3'-UTR.

CONCLUSIONS: This study showed that miR-30c overexpression inhibited CRC proliferation via the regulation of BCL9, suggesting that miR-30c may be a new molecular therapeutic target for CRC.

Key Words:
miR-30c, Colorectal carcinoma, BCL9.

Introduction

Colorectal carcinoma (CRC) is one of the most common malignancies with high mortality, and CRC is the prominent cause of cancer-related death globally[1]. As mostly diagnosed at advanced stage, the death rate of CRC remains high[2]. So if diagnosed at early stage, the prognosis of CRC patients could be improved. Recently, to detect CRC accurately, colonoscopy is in widespread application although it has lots of deficiencies[3]. In addition, the pathogenesis and the factors for the high relapse rate of CRC remain unclear[4]. Therefore, up-to-date and reliable biomarkers for early diagnosis of CRC are needed. More and more researches have focused on microRNAs (miRNAs/miRs), the aberrant expression of which was reported to promote or suppress the development of multiple cancers. miRNAs are small noncoding RNAs that have been found to be involved in kinds of biological processes. miRNA plays a crucial role in regulating gene expression by complementary binding to the 3'-untranslated region (3'-UTR) of target mRNA[5]. For example, miR-494 was reported to suppress tumor growth of epithelial ovarian carcinoma by targeting insulin-like growth factor 1 receptor (IGF1R)[6]; miR-345 could repress hepatocellular carcinoma metastasis by inhibiting yes-associated protein 1(YAP1)[7]; miR-374b suppressed proliferation and promoted apoptosis in T-cell lymphoblastic lymphoma by repressing protein kinase B (AKT1) and Wnt-16[8]. These findings demonstrated the involvement of miRNAs in the pathogenesis of cancers, and miRNAs may function as effective biomarkers to predict outcomes and improve therapy responses of patients with different cancers. However, little is known about the biological functions of miR-30c in CRC progression and treatment. MiR-30 family has been recently shown to be involved in pathophysiological events by regulating common target genes[9]. MiR-30c, a member of the miR-30 family, is expressed in numerous types of malignant tumors. For instance, miR-30c was downregulated in squamous cell carcinoma of the vulva[10]; miR-30c suppressed prostate cancer survival by targeting the oncoprotein alternative splicing factor (ASF)/splicing factor 2 (SF2)[11]; miR-30c-5p

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suppressed gastric cancer migration, invasion and epithelial to mesenchymal transition *via* targeting metastasis-associated gene 1 (MTA1)[12]. Although miR-30c studies predominate in the cancer fields, little is known about its functions in CRC. B cell lymphoma 9 (BCL9), one component of the Wnt/ β-catenin pathway, has emerged as a novel target for cancer therapy[13,14]. BCL9 was overexpressed in a variety of malignancies and promoted cell proliferation, invasion and metastasis[15,16]. Jia et al[17] have identified BCL9 as a direct target of miR-30c in a number of cancers. However, a functional link between miR-30c and BCL9 in CRC has not been established. We aimed to investigate the functions of miR-30c and BCL9 in CRC development.

Patients and Methods

Human CRC Tissue Samples and Cell Lines

Fifty pairs of CRC samples and matched adjacent non-tumor tissues were collected from Yantai Yuhuangding Hospital between 2015 and 2017. The demographics and clinicopathological characteristics of the CRC patients were shown in Table I. The tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. All patients provided written informed consent and this study was approved by the Ethics Committee of Yantai Yuhuangding Hospital. Normal intestinal epithelial cell line CCD-18Co and human CRC cell lines LOVO and SW480 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a humidified incubator containing 5% CO_2 at 37°C.

Cell Transfection

MiR-30c mimics, miR-30c inhibitor or BCL9-siRNA and the corresponding controls were transfected into CRC cell lines using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

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

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from the tissues and cell lines. Complementary Deoxyribose Nucleic Acid (cDNA) was synthesized using the PrimeScript RT reagent kit (TaKaRa

Table I. Correlation of miR-30c expression with the clinicopathological characteristics of the colorectal carcinoma patients.

Clinicopathological features	Cases (n=50)	miR-30c expression		*p*-value
		High (n=15)	Low (n=35)	
Age (years)				0.5748
> 60	24	6	18	
≤ 60	26	9	17	
Gender				0.3357
Male	24	8	16	
Female	26	7	19	
Tumor size (cm)				0.2544
≥ 5.0	25	5	20	
< 5.0	25	10	15	
TNM stage				0.0061
I-II	17	11	6	
III	33	4	29	
Lymph-node metastasis				0.0058
Yes	30	3	27	
No	20	12	8	
Location				0.4795
Colon	22	7	15	
Rectum	28	8	20	
Distant metastasis				0.5204
Yes	25	6	19	
No	25	9	16	

TNM: tumor-node-metastasis.

Biotechnology Co., Ltd., Dalian, China) following the manufacturer's instructions. Quantitative Real-time PCR (qRT-PCR) was performed with the SYBR Premix Ex Taq kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) on an ABI 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as endogenous controls. The relative quantitative expression levels were calculated through the relative quantification $2^{-\Delta\Delta CT}$ method. The primers used were as follows: miR-30c forward: 5'-TGT GTT TTT ATT GTT TTT GTT GTC CCA-3' and reverse: 5'-GGG ACA GAA CAG GTT AAT GGG AA-3'; BCL9 forward: 5'-AGG GAG CGA AGT ATT TCC GC-3' and reverse: 5'-GGG TCA TCG AGT GTG GTG TG-3'; U6 forward: 5'-CGC TTC GGC AGC ACA TAT AC-3' and reverse: 5'-CAG GGG CCA TGC TAA TCT T-3'; GAPDH forward: 5'-CGT GGG CCG CCC TAG GCA CCA-3' and reverse: 5'-TTG GCT TAG GGT TCA GGG GGG-3'.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetraz-Olium Bromide (MTT) Assay

Cell proliferation was determined by MTT cell proliferation kit (Solarbio, Beijing, China). CRC cell lines (LOVO and SW480) transfected with miR-30c mimics, inhibitors, siRNAs or corresponding controls were seeded in 96-well plates. MTT solution was added to each well and incubated at 37°C with 5% CO_2. After incubating for 12, 24, 48 and 72 h, the MTT solution was removed. Next, dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for another 10 min. The absorbance at 570 nm was measured using a microplate reader.

Luciferase Assay

The synthetic mutant (MUT) or wild-type (WT) BCL9-3'-UTR fragment containing the conserved miR-30c binding sites was inserted into luciferase reporter plasmids. CRC cell lines (LOVO and SW480) were cotransfected with miR-30c mimics and BCL9-3'UTR-WT or corresponding mutant reporter using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was measured 48 h post-transfection using dual-luciferase reporter system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical Analysis

All data were obtained from at least three independent experiments. The results are presented as the means ± standard deviation (SD). The statistical analysis was performed with Statistical Product and Service Solutions (SPSS) 18.0 version (SPSS Inc. Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Student's *t*-test was used in the present study. Correlation between mRNA and miRNA was estimated using the Spearman's correlation method. $p<0.05$ was regarded as statistically significant difference.

Results

Low Expression of miR-30c and Overexpression of BCL9 were Identified in Colorectal Carcinoma

To better understand the functions of miR-30c in CRC, firstly, we examined the expression of miR-30c in 50 CRC tissue samples and adjacent normal tissues using qRT-PCR. The results showed that, compared to adjacent normal tissues, CRC tissues presented significantly decreased miR-30c expressions (Figure 1A, $p<0.01$). The same result was also found in CRC cell lines. More specifically, qRT-PCR was performed to measure miR-30c expression levels in CRC cell lines (LOVO and SW480) and normal intestinal epithelial cell line. Experimental findings manifested lower miR-30c expressions in LOVO and SW480 cells than the normal cell line (Figure 1B, $p<0.01$). We also measured BCL9 mRNA expression in CRC cell lines, and the qRT-PCR results demonstrated significantly increased BCL9 expressions in CRC cell lines compared to the control group (Figure 1C, $p<0.01$, $p<0.001$). Moreover, we analyzed the correlation between miR-30c and BCL9 expression in CRC tissues and found a negative correlation between them (Figure 1D, $p<0.001$). Additionally, CRC patients who had a high expression level of miR-30c showed higher overall survival rates than those who had a low expression level of miR-30c (Figure 1E, $p<0.05$).

MiR-30c Inhibited Cell Proliferation in Colorectal Carcinoma Cell Lines

MTT assay was performed to verify the role of miR-30c in regulating the proliferation of CRC cells. Firstly, miR-30c overexpression or knockdown models were established by transfecting miR-30c mimics or inhibitor into the CRC cells. Results showed a significantly higher miR-30c expression level in CRC cells transfected with miR-30c mimics than the control

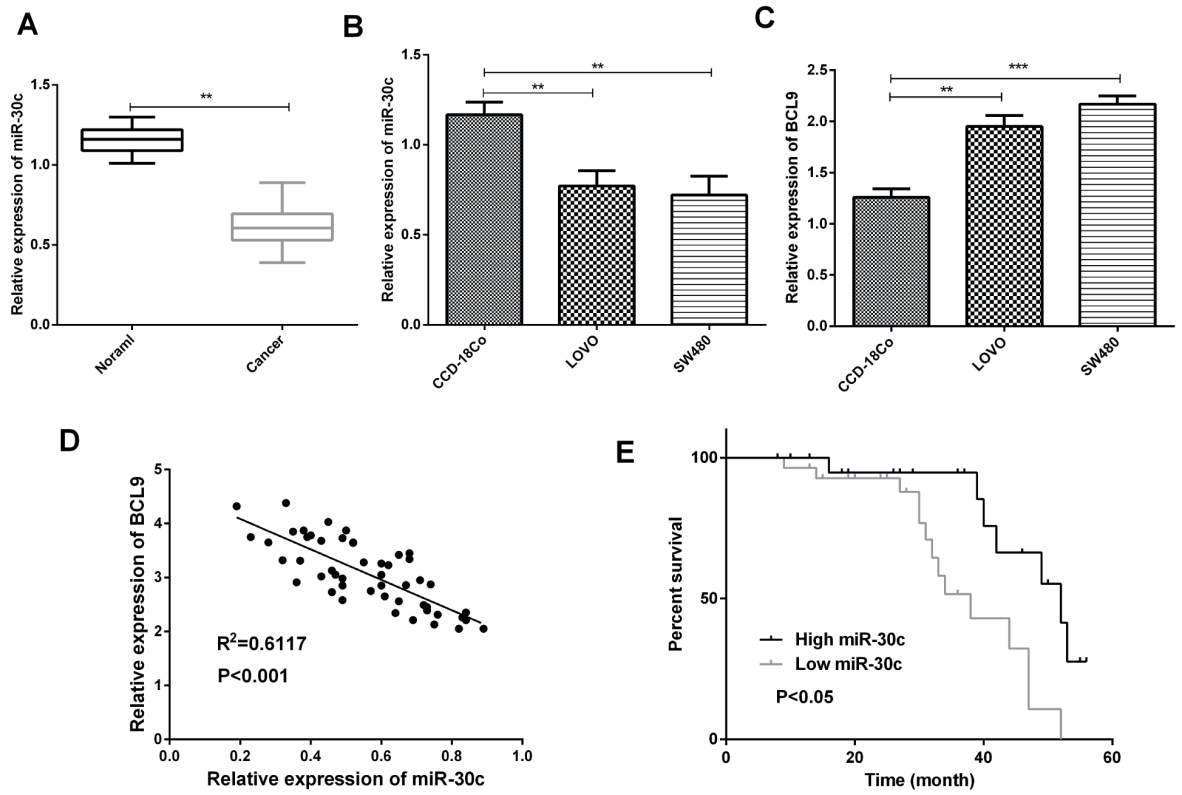

Figure 1. MiR-30c was downregulated and BCL9 was upregulated in colorectal carcinoma tissues and cell lines. ***A,*** miR-30c expression was analyzed by qRT-PCR in colorectal carcinoma tissues (n=50) and adjacent normal tissues (n=50) (***p<0.001). ***B,*** qRT-PCR analysis of miR-30C expression level in CRC cell lines (LOVO and SW480) and the normal intestinal epithelial cell line CCD-18Co. ***C,*** The mRNA expression level of BCL9 was detected by qRT-PCR in CRC cell lines (LOVO and SW480) (**p<0.01). ***D,*** Spearman's correlation analysis of miR-30c and BCL9 expressions in colorectal carcinoma tissues. ***E,*** miR-30c expression level was associated with overall survival of colorectal carcinoma patients.

group; oppositely, the miR-30c expression level in CRC cells transfected with miR-30c inhibitor was significantly reduced when compared to the control group (Figure 2A and 2B, p<0.01). The effect of miR-30c on cell proliferation was investigated in LOVO and SW480 cells transfected with miR-30c mimics or miR-30c inhibitor, respectively. The results of MTT assays indicated that the miR-30c overexpression distinctly inhibited cell proliferation in LOVO (Figure 2C) and SW480 (Figure 2D) cells.

MiR-30c Regulated BCL9 Expression Through Targeting its 3'-UTR

To explore the possible target genes of miR-30c, TargetScan was used and the searching result identified BCL9 as a putative target of miR-30c (Figure 3A). To further confirm the relationship between miR-30c and BCL9, luciferase reporter assay was performed in CRC cells by cotransfecting with miR-30c mimics and the luciferase reporter plasmids containing WT or MUT BCL9 3'UTR severally. Finally, we found that the luciferase activity in LOVO and SW480 cells which were cotransfected with BCL9-3'UTR-WT and miR-30c mimics was significantly decreased when compared to the control group. However, there was no distinct difference between the CRC cells cotransfected with the BCL9-3'UTR-MUT luciferase reporter plasmid and miR-30c mimics and the corresponding control cells (Figure 3B and 3C, p<0.01). Furthermore, we detected the expression of BCL9 in LOVO and SW480 cells transfected with miR-30c mimics or miR-30c inhibitor. In the cells transfected with miR-30c mimics, there was a significant decrease in the expression level of BCL9 compared to the cells transfected with the control mimics. On the contrary, the expression level of BCL9 in CRC

cells transfected with miR-30c inhibitor was significantly higher than the matched control group (Figure 3D and 3E, $p<0.01$).

BCL9 Regulated the Function of miR-30c in Colorectal Carcinoma Cell Proliferation

To confirm the role of BCL9 in regulating the function of miR-30c in colorectal carcinoma cell proliferation, LOVO and SW480 cells were transfected with specialized BCL9 siRNA, which could knockdown the endogenic BCL9. Then, the mRNA expression level of BCL9 was measured by qRT-PCR. The results indicated that BCL9 was markedly downregulated by BCL9 siRNA in both LOVO and SW480 cells compared to the cells transfected with control siRNA (Figure 4A and 4B, $p<0.01$). Moreover, MTT assays were conducted to investigate the proliferation abilities of LOVO and SW480 cells cotransfected with BCL9 siRNA and miR-30c inhibitor. Our study provided evidence that deletion of BCL9 significantly altered the inhibition function of miR-30c in CRC cell proliferation (Figure 4C and 4D).

Discussion

Nowadays, CRC is not only an important problem in clinical practice but also a critical challenge for public health. Although recent advances in medicine have improved the survival of CRC patients, CRC remains a major public health problem due to various contributory factors[18]. Recently, multiple miRNAs have been increasingly demonstrated to play crucial roles in gene regulation in CRC. For example, miR-592 exerted oncogenic role in CRC tum-

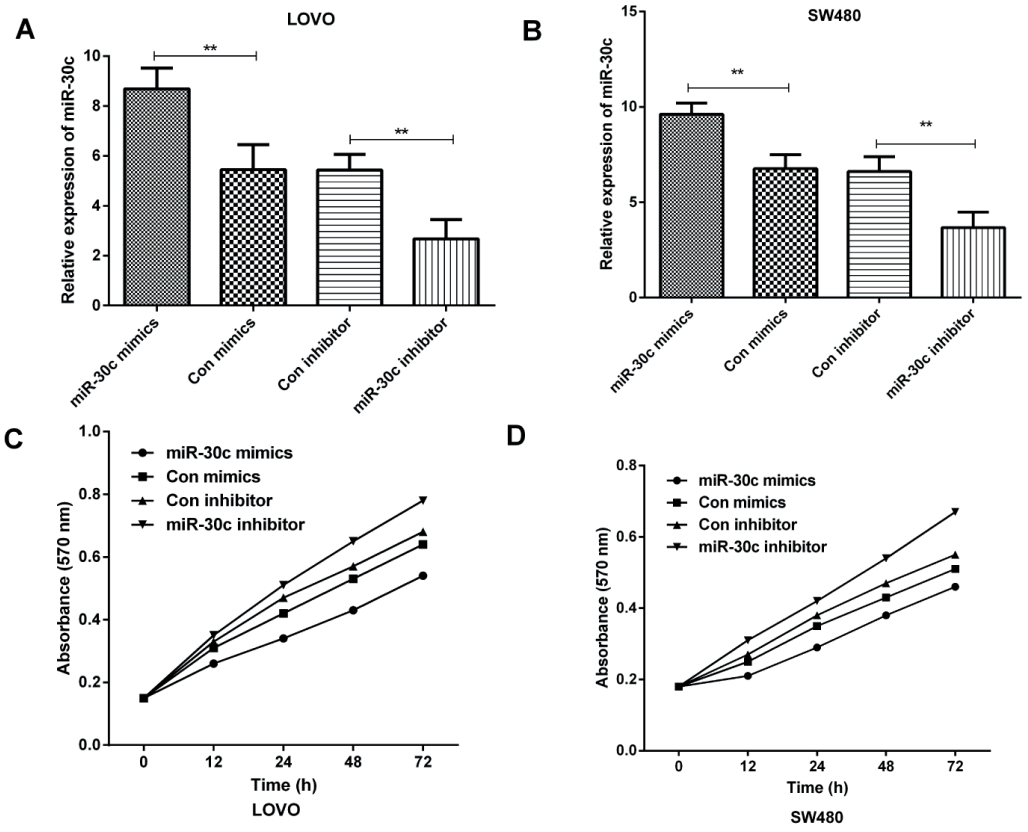

Figure 2. MiR-30c inhibited cell proliferation in colorectal carcinoma cell lines. ***A,*** The expression level of miR-30c was measured by qRT-PCR in LOVO cells transfected with miR-30c mimics or miR-30c inhibitor, respectively (**$p<0.01$, *$p<0.05$). ***B,*** The expression level of miR-30c was measured by qRT-PCR in SW480 cells transfected with miR-30c mimics or miR-30c inhibitor, respectively (**$p<0.01$, *$p<0.05$). ***C,*** The MTT assay was performed to detect proliferation of LOVO cells transfected with miR-30c mimics or miR-30c inhibitor, respectively, at 0, 12, 24, 48 and 72 h. ***D,*** The MTT assay was performed to detect proliferation of SW480 cells transfected with miR-30c mimics or miR-30c inhibitor, respectively, at 0, 12, 24, 48 and 72 h.

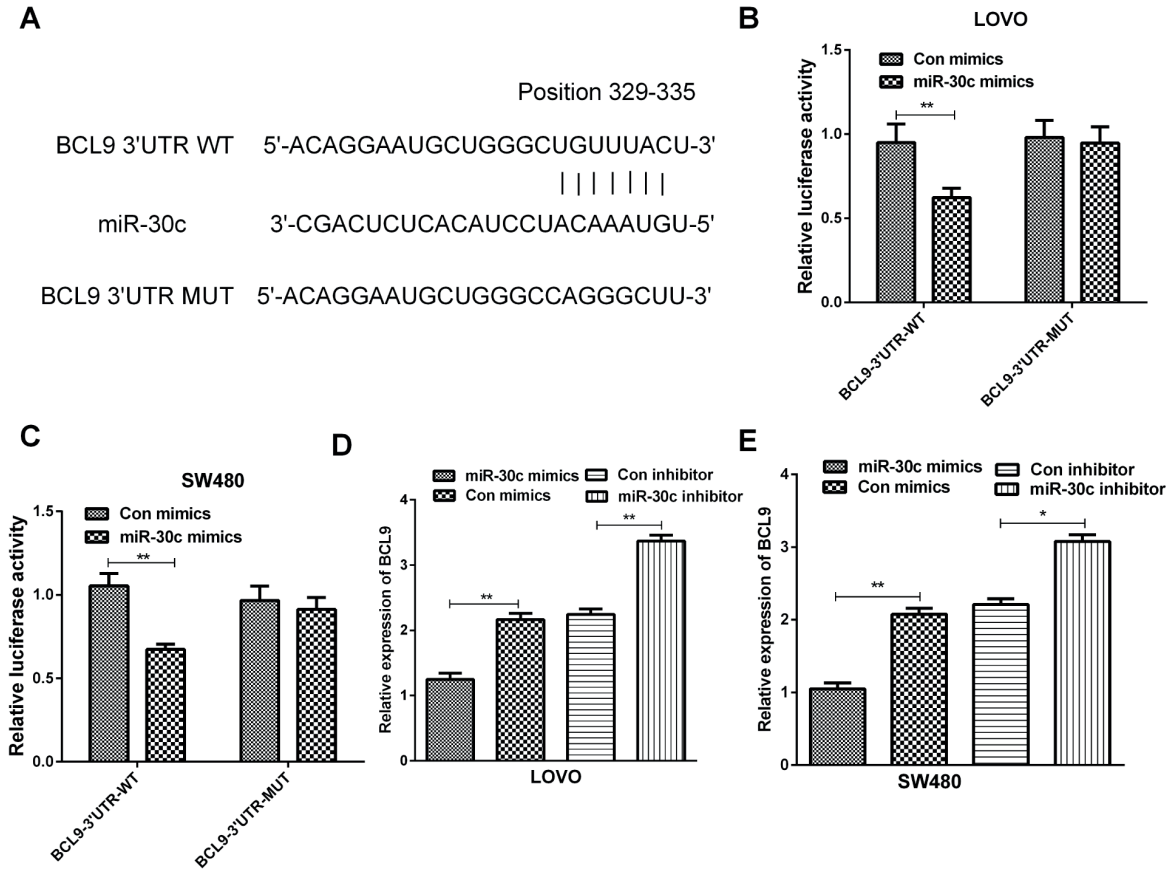

Figure 3. MiR-30c regulated BCL9 expression through targeting its 3'-UTR. ***A,*** According to the Target Scan, the miR-30c target site in the sequence of BCL9 was predicted. ***B,*** The fluorescence activity was measured by the luciferase reporter gene assay in LOVO cells that were cotransfected with BCL9 3'UTR-WT or BCL9 3'UTR-MUT and miR-30c mimics, respectively (**p<0.01). ***C,*** The fluorescence activity was measured by the luciferase reporter gene assay in SW480 cells that were cotransfected with BCL9 3'UTR-WT or BCL9 3'UTR-MUT and miR-30c mimics, respectively (**p<0.01). ***D,*** qRT-PCR was used to determine the mRNA expression level of BCL9 in LOVO cells transfected with miR-30c mimics or inhibitor, respectively (***p<0.001). ***E,*** qRT-PCR was used to determine the mRNA expression level of BCL9 in SW480 cells transfected with miR-30c mimics or inhibitor, respectively (***p<0.001).

origenesis by targeting forkhead box transcription factor class O3a (FoxO3A)[19]; miR-875-5p functioned as a tumor suppressor function in CRC through downregulation of epidermal growth factor receptor (EGFR)[20]; miR-17-92 inhibited CRC progression by targeting angiogenesis[21]. However, the potential effects of miR-30c on regulating CRC remain unclear. Previous studies demonstrated that the aberrant expression of miR-30c was related to the progression of multiple cancers, such as breast cancer[22], non-small cell lung cancer[23], gastric cancer[24]. Although miR-30c has been widely regarded as a tumor suppressor in a variety of cancers, its functions in CRC remain unknown due to the lack of relatively investigations. Moreover, the mechanisms underlying the tumor-suppressive role of miR-30c have not been well described. Current study indicated that the expression level of miR-30c in CRC was prominently decreased and miR-30c overexpression inhibited CRC proliferation partially *via* the regulation of its direct target BCL9. Aberrant expression of BCL9 is an oncogenic mechanism of Wnt pathway activation[25]. The BCL9 gene is a good candidate gene for cancers because it is a functional component of the Wnt signaling pathway, which plays a crucial role in a number of developmental processes, such as directing growth and cell development [26,27]. Earlier stud-

ies indicated that the tumor suppressor miR-30c was involved in PCa pathogenesis by targeting BCL9[28]. Present finding supported the notion that BCL9 may have potential as a biomarker for CRC, and that targeting aberrant levels of BCL9 should be explored as a potential approach to improve clinical outcomes.

Conclusions

We measured the expression level of miR-30c and BCL9 in CRC tissues and cells, respectively, and we demonstrated that miR-30c was significantly decreased in CRC tissues and cell lines while the BCL9 expression level was remarkably increased. In addition, we analyzed the correlation between the miR-30c and BCL9 expressions in CRC tissues, and a negative correlation between them was identified. Furthermore, the findings of this study also showed that BCL9 was a direct target of miR-30c in CRC and miR-30c could inhibit the CRC cell proliferation by binding to its 3'-UTR. This study is likely important in inhibiting CRC and may act as a novel therapeutic option for patients with CRC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

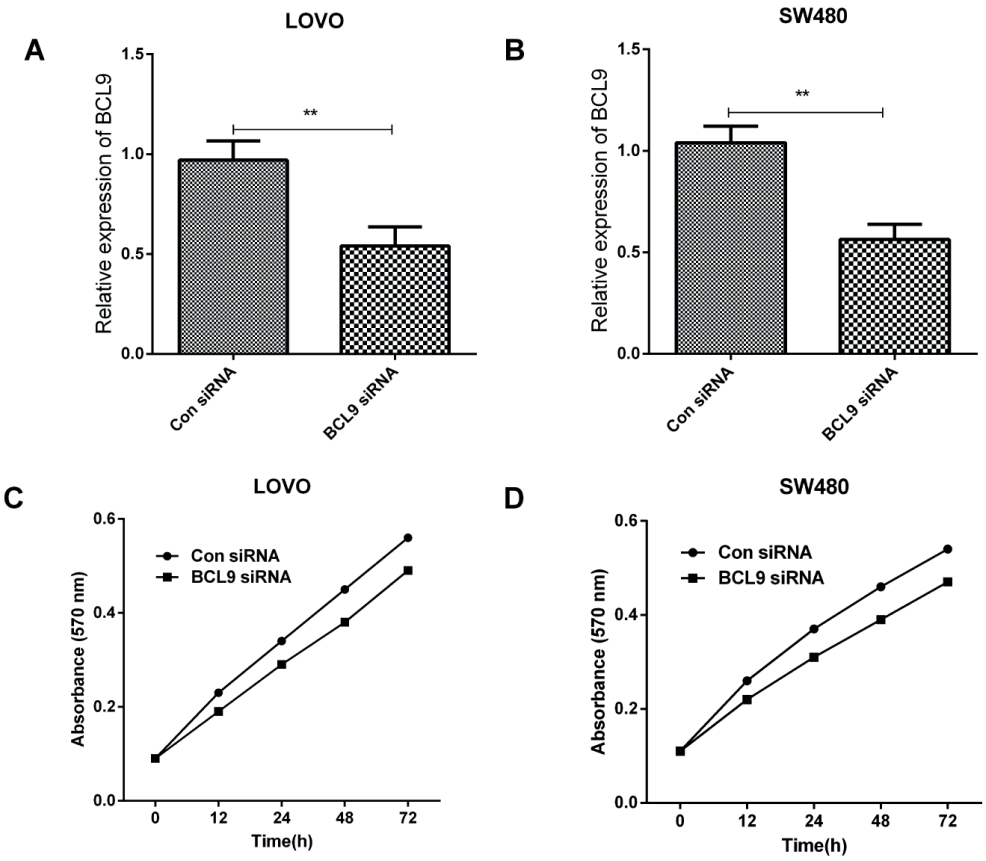

Figure 4. BCL9 regulated the function of miR-30c in colorectal carcinoma cell proliferation. ***A,*** The mRNA expression level of BCL9 in LOVO cells transfected with BCL9 siRNA was detected using qRT-PCR (**p<0.01). ***B,*** The mRNA expression level of BCL9 in SW480 cells transfected with BCL9 siRNA was measured using qRT-PCR (**p<0.01). ***C,*** The MTT assay was performed to detect the proliferation ability of LOVO cells cotransfected with BCL9 siRNA and miR-30c inhibitor at 0, 12, 24, 48 and 72 h. ***D,*** The MTT assay was performed to detect the proliferation capacity of SW480 cells cotransfected with BCL9 siRNA and miR-30c inhibitor at 0, 12, 24, 48 and 72 h.

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