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MiR-3174 functions as an oncogene in rectal cancer by targeting PCBD2

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Abstract. – OBJECTIVE: Colorectal cancer (CRC) is one of the most frequent malignant tumors worldwide. Colon cancer and rectal cancer are two different malignant types, and it is important to distinguish these two cancers. However, the physiological function of microRNA-3174 (miR-3174) in rectal cancer remains unknown. Therefore, the aim of this study was to investigate the role of miR-3174 in rectal cancer progression and to explore the possible underlying mechanism.

PATIENTS AND METHODS: The relative expression level of miR-3174 in rectal cancer was evaluated by quantitative Real-time polymerase chain reaction (qRT-PCR). Cell counting kit-8 (CCK8) assay and colony formation assay were employed to detect the proliferation ability of cells. Flow cytometric analysis was used to detect cell cycle distribution and apoptotic cells. Bioinformatics analysis and dual luciferase reporter gene assay were employed to predict and verify the target genes of miR-3174, respectively. Meanwhile, the protein expression level of pterin-4 alpha-carbinolamine dehydratase 2 (PCBD2) normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assessed by Western blotting.

RESULTS: The expression level of miR-3174 was significantly up-regulated in rectal cancer. CCK8 assay and colony formation assay suggested that miR-3174 markedly promoted the proliferation of rectal cancer cells. Subsequently, flow cytometric analysis demonstrated that over-expressed miR-3174 significantly accelerated cell cycle, whereas remarkably inhibited cell apoptosis. Public prediction websites and dual luciferase reporter gene assay further validated that PCBD2 was a direct down-stream target of miR-3174. Moreover, rescue assay confirmed that miR-3174 functioned as an oncogene in rectal cancer by regulating PCBD2.

CONCLUSIONS: Our study elucidated that miR-3174 functioned as an oncogene in rectal cancer by targeting PCBD2, which might bring new insights into the search for novel biomarkers and therapeutic strategies.

Key Words:
MiR-3174, Proliferation, Apoptosis, PCBD2.

Introduction

Colorectal cancer (CRC) is the third most frequent cancer over the world. It is reported that there were more than 1.36 million new cases in 2012, 30% of which were rectal cancer[1]. CRC remains the fourth leading cause of cancer-related mortality and morbidity[2]. Colon cancer and rectal cancer are two distinct cancer types, meanwhile, the treatment strategies of these two cancers are totally different[3]. The survival of colon cancer and rectal cancer differs. Meanwhile, fewer inherited syndromes and younger age are found in patients with rectal cancer when diagnosed[4]. Thus, it is essential to separate these two types of cancers. The main purpose of distinguishing between the two cancers is anatomical and molecular differences[5,6]. Hence, it is helpful to search novel biomarkers and treatment strategies. MicroRNAs (miRNAs) are a class of short non-coding RNAs with 20-22 nucleotides in length. MiRNAs were first discovered in 2001[7]. Studies have proved that miRNAs can lead to post-transcriptional gene silencing through inducing mRNA degradation or transcriptional inhibition[8]. MiRNAs play an important role in various physiological and pathophysiological processes, including cell proliferation, cell apoptosis, cell cycle, cell differentiation and metabolism[8,9]. Previous researches[10] have found that miR-3174 contributes to cell apoptosis and autophagy cell death defects in gastric cancer by regulating ARHGAP10. However, the exact role of miR-3174 in rectal cancer progression remains unclear. Herein, the aim of this study was

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to investigate the physiological role of miR-3174 in rectal cancer as well as the possible underlying mechanism. MiRNAs are known to exert their physiological functions by pairing with the 3'UTR region of down-stream targets and regulating target gene expression[11]. Thus, we used bioinformatics analysis to predict the potential target gene of miR-3174. Results showed that pterin-4 alpha-carbinolamine dehydratase 2 (PCBD2) was an underlying target gene of miR-3174. Further dual luciferase reporter gene assay confirmed our hypothesis. Hence, we speculated that miR-3174 might exert its physiological function in rectal cancer by regulating PCBD2. In this research, quantitative real-time polymerase chain reaction (qRT-PCR) results found that miR-3174 was significantly upregulated in rectal cancer. Cellular functional assays proved that miR-3174 could significantly promote rectal cancer cell proliferation and inhibit cell apoptosis. Besides, the results of bioinformatics analysis and dual luciferase reporter gene assay confirmed that miR-3174 functioned as an oncogene in rectal cancer by regulating PCBD2. Our study might bring a novel insight of biomarkers and treatment strategies for rectal cancer.

Patients and Methods

Tissue Specimens

This study was approved by the Ethics Committee of Beijing Chaoyang Hospital, Capital Medical University. Informed consent was obtained from each subject before the study. 30 pairs of rectal cancer tissues and normal tissues were collected from patients who received treatment in Beijing Chaoyang Hospital, Capital Medical University from 2016 to 2017. All collected tissues were immediately preserved in liquid nitrogen for subsequent experiments.

Cell Culture

Rectal cancer cell lines SW837 and SW1463 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Human rectal mucosa epithelium cell (PriCells, Wuhan, China) was used as a normal control (NC). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, HyClone, South Logan, UT, USA) medium containing 10% fetal bovine serum (FBS Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C, 5% CO_2 incubator.

Cell Transfection

To up-regulate or down-regulate the expression of miR-3174 in selected rectal cancer cell lines, we constructed lentivirus vectors and empty lentivirus vector control (GenePharma, Shanghai, China). All transfected cells were screened by puromycin. SiRNA transfection reagent (Invitrogen, Carlsbad, CA, USA) was used to down-regulate the expression level of PCBD2 in accordance with the instructions. Transfection efficiency was determined by qRT-PCR.

RNA Extraction And Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in tissues and cell lines was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The Reverse Transcription Kit (TaKaRa, Dalian, China) was used to synthesize complementary DNAs (cDNAs). The relative expression levels of miR-3174 and PCBD2 were detected by qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. QRT-PCR was operated in the ABI 7500 system (Applied Biosystems, Foster City, CA, USA). Specific qRT-PCR reaction conditions were: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. Relative expression level of target gene was calculated by the $2^{-\Delta\Delta Ct}$ method. Primers used in this study were as follows: PCBD2, F: 5'-ACTTCTCCACAATTCTCTG -3', R: 5'-CTAGGGCCGTGTCCGAAGGA-3'; microRNA-3174, F: 5'-GTCAGGGATGGCAACTTTATCCACT-3', R: 5'- GGAACCTGAAGGTCCGAGTCA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Colony Formation Assay

A total of 1.0×10^3 cells were planted into 60 mm culture plates and cultured for 2 weeks. After washing with phosphate-buffer saline (PBS) (Beyotime, Shanghai, China) twice, the cells were fixed with ice-cold 70% methanol for 15 min. Subsequently, formed colonies were stained with Crystal Violet Staining Solution (Beyotime, Shanghai, China). All the colonies were captured, and the number of colonies was finally counted.

Cell-Counting Kit-8 Assay

To examine the proliferation ability of cells, we performed cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). Transfected cells were first planted into 96-wells plates at a density of 6×10^3/well. 10 uL CCK-8 solution (Beyotime, Shanghai, China) (/well) was added to each well, followed by incubation at 37°C for 2 h in dark. The OD value (450 nm) was detected by a spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Flow Cytometry

To detect cell apoptosis, flow cytometry was performed according to the instructions of Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, Nanjing, China). To examine cell cycle, transfected cells were immersed in cold 70% ethanol overnight and stained with propidium iodide (PI) (Vazyme, Nanjing, China). Flow cytometry was conducted by BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA).

Bioinformatics Analysis

In this study, 4 public available databases were used to predict the underlying target genes of miR-3174, including RNA22, TargetScan, miRWalk and MiRanda. Results suggested that 3'-UTR of PCBD2 had potential binding sites with miR-3174. Thus, we considered that PCBD2 might be a direct down-stream target of miR-3174.

Luciferase Report Gene Assay

Wild-type PCBD2 3'-UTR sequence or the mutant PCBD2 3'-UTR sequence was combined with pGL3 promoter vector (Genscript, Nanjing, China). MiR-3174 mimics or negative control oligo-ribonucleotides were purchased from GenePharma (Shanghai, China). Luciferase activity was determined according to the instructions of Victor 1420 Multilabel Counter (Wallac, Finland) by Luciferase Assay System (Promega, Madison, WI, USA).

Western Blot

Total protein was extracted by radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher, Waltham, MA, USA) and Phenylmethanesulfonyl fluoride (PMSF) (Thermo Fisher, Waltham, MA, USA). Extracted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Then, the membranes were incubated with primary antibody of rabbit anti-PCBD2 (1:1000, CST, Danvers, MA, USA) at 4°C overnight. Rabbit anti-GAPDH (1:5000, CST, Danvers, MA, USA) was taken as a loading control. Relative protein expression level was evaluated by Image J software.

Statistical Analysis

All experiments in this study were repeated for at least three times. All data were represented as mean ± standard deviation (SD). Pearson correlation analysis was performed to analyze the correlation between miR-3174 and PCBD2. Student's unpaired *t*-test was used to compare the differences between two groups. $p < 0.05$ was considered statistically significant.

Results

MiR-3174 Was Up-Regulated in Rectal Cancer

We first detected the relative expression level of miR-3174 in rectal cancer tissues and normal tissues by qRT-PCR. A total of 30 paired rectal cancer tissues and normal tissues were selected in this study. As shown in Figure 1A, the relative expression level of miR-3174 in rectal cancer tissues was significantly higher than that of normal tissues. Consistently, the expression of miR-3174 was significantly up-regulated in rectal cancer cell lines (Figure 1B). According to the expression level of miR-3174 in SW837 and SW1463 cells, they were transfected with lentivirus vectors for over-expressing or down-expressing miR-3174. Transfection efficiency was examined by qRT-PCR (Figure 1C). Based on these findings, we suggested that miR-3174 was over-expressed in rectal cancer.

Up-Regulated miR-3174 Promoted the Proliferation of Rectal Cancer Cells

We then performed CCK-8 assay to detect the proliferation ability of transfected cells. As shown in Figure 2A, when compared with the control group, the miR-3174 overexpression group had significantly higher OD values in SW837 cells. However, the ANTI down-regulation group had obviously lower OD values in SW1463 cells. Furthermore, we detected the colony formation ability of transfected cells. As shown in Figure 2B, miR-3174 overexpression significantly accelerated the colony formation ability, while miR-3174 down-regulation remarkably the col-

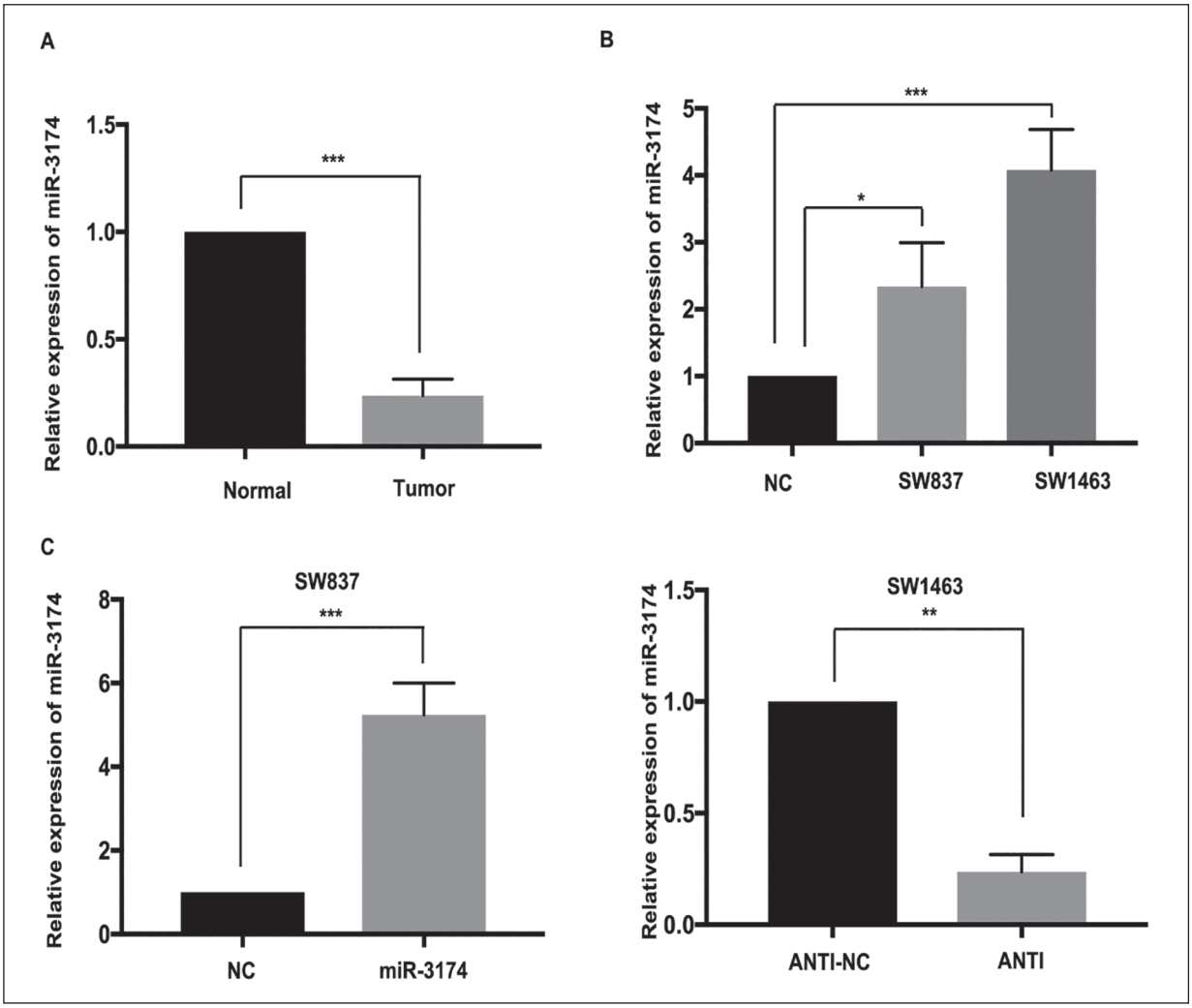

Figure 1. Expression level of miR-3174 in rectal cancer tissues and cell lines. ***A,*** The relative expression level of miR-3174 in 30 paired rectal cancer tissues and normal tissues was detected. ***B,*** QRT-PCR was used to detect the expression of miR-3174 in rectal cancer cell lines. ***C,*** Expression of miR-3174 in SW837 cells after transfection with lentivirus vector for over-expressing miR-3174. ***D,*** Expression of miR-3174 in SW1463 cells after transfection with lentivirus vector for down-expressing miR-3174. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the control group. Data were expressed as mean ± SD.

ony formation ability. Thus, we considered that up-regulated miR-3174 could promote th eproliferation of rectal cancer cells.

Up-Regulated miR-3174 Accelerated Cell Cycle and Inhibited Cell Apoptosis in Rectal Cancer

To explore the effect of miR-3174 on cell cycle and cell apoptosis, we employed flow cytometry. As exhibited in Figure 3A, the distribution of G1/G0 phase was significantly decreased in the miR-3174 group, while the distribution of S phase was increased. Opposite results were found in the ANTI group. Thus, we speculated that up-regulated miR-3174 could accelerate cell cycle of rectal cancer cells. Flow cytometry was subsequently used to detect the apoptotic rate of cells. As shown in Figure 3B, the apoptotic rate in the miR-3174 group was significantly lower than that of the NC group. However, the apoptotic rate of the ANTI group was significantly higher than the ANTI-NC group. In sum, we considered that up-regulated miR-3174 could accelerate cell cycle and inhibit cell apoptosis in rectal cancer cells.

PCBD2 was a Direct Down-Stream Target of miR-3174

To forecast the underlying down-stream target of miR-3174, we searched three public available databases and found that that the 3'UTR of

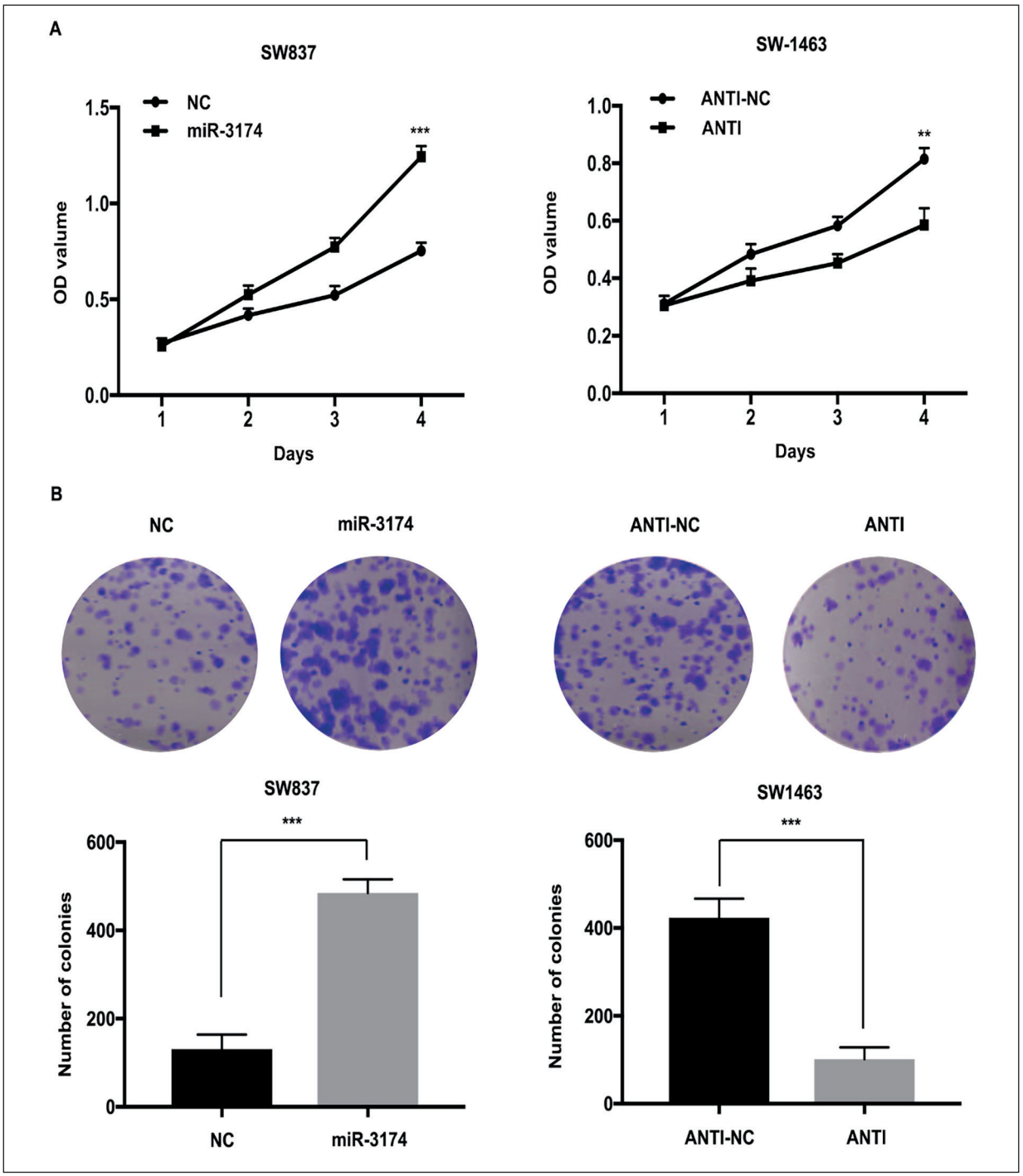

Figure 2. MiR-3174 promoted the proliferation of rectal cancer cells. ***A,*** CCK-8 was used to analyze cell proliferation ability. Higher OD values represented more cell proliferation. ***B,*** Cell colonies were stained with crystal violet, and the number of colonies was counted. The time for cell colony formation was 10 days. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control group. Data were expressed as mean ± SD.

PCBD2 had potential binding site with miR-3174. Subsequently, dual luciferase reporter gene assay was performed to validate our hypothesis. As shown in Figure 4A, the luciferase activity of cells co-transfected with wild-type PCBD2 3'UTR and mimics was significantly lower than of the control group. Thus, we believed that PCBD2 was a down-stream target of miR-3174.

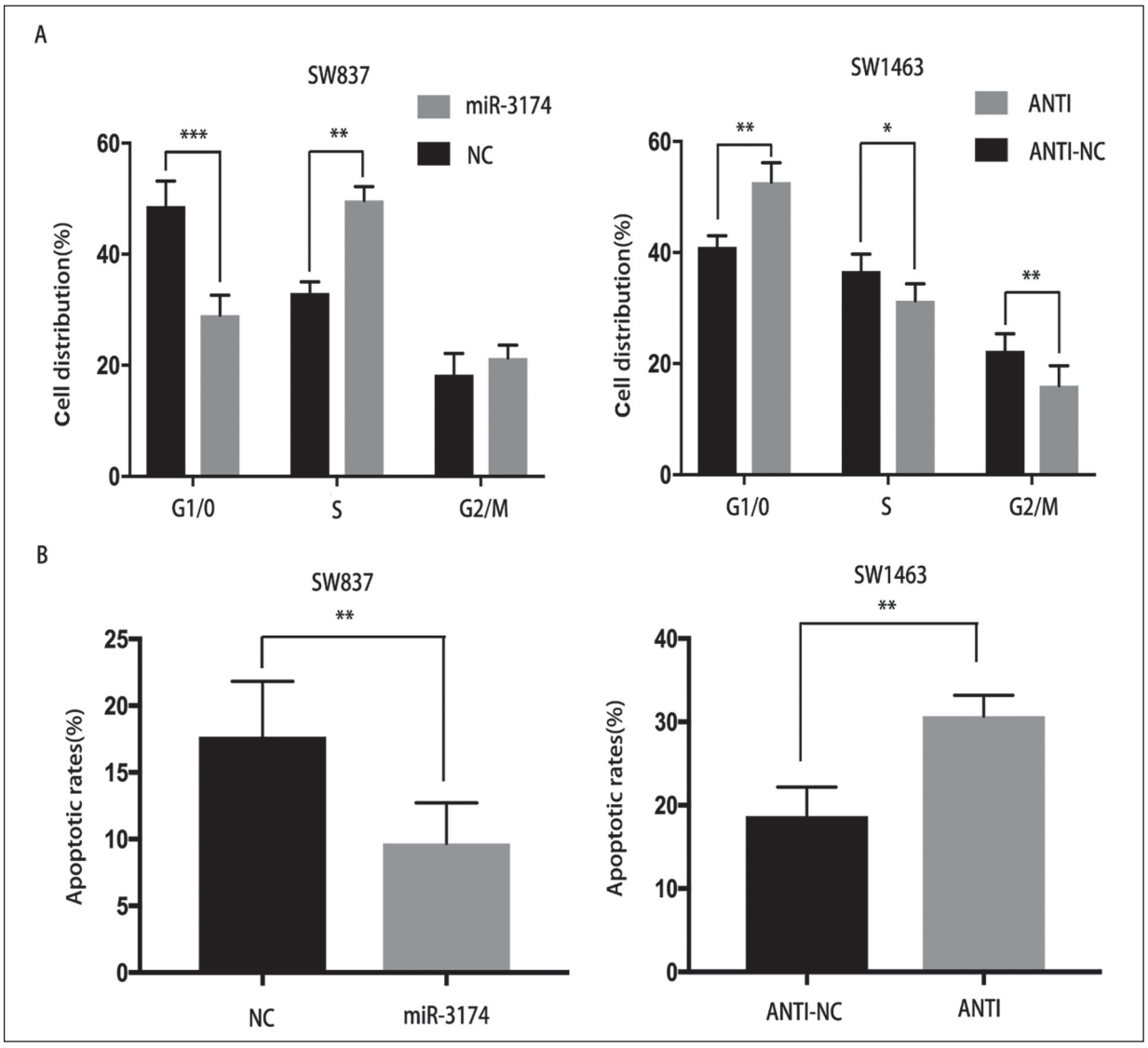

Figure 3. MiR-3174 accelerated cell cycle progression and inhibited cell apoptosis. ***A,*** Cell cycle was detected by flow cytometry. ***B,*** The rate of living cells was examined by flow cytometry. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the control group. Data were expressed as mean ± SD.

Furthermore, we conducted qRT-PCR to examine the expression level of PCBD2 in tissues. Results showed that the expression of PCBD2 in rectal cancer tissues was significantly down-regulated (Figure 4B). Pearson correlation analysis results indicated that miR-3174 was negatively correlated with PCBD2 in rectal cancer (Figure 4C). As shown in Figure 4D-4E, the expression of PCBD2 was down-regulated in the miR-3174 group, whereas the expression of PCBD2 was up-regulated in the ANTI group. Taken together, we suggested that PCBD2 was a direct downstream target of miR-3174.

MiR-3174 Promoted Cell Proliferation and Suppressed Cell Apoptosis By Regulating PCBD2 In Rectal Cancer

To verify whether miR-3174 played an oncogenic role in rectal cancer by regulating PCBD2, we subsequently performed rescue assay. As shown in Figure 5A, transfection efficiency of siRNAs was detected by qRT-PCR. In the rescue assay, we co-transfected si-PCBD2-NC or si-PCBD2 with lentivirus vector in the ANTI group. Meanwhile, the co-transfection efficiency was also determined by qRT-PCR (Figure 5B). As shown in Figure 5C, colony formation

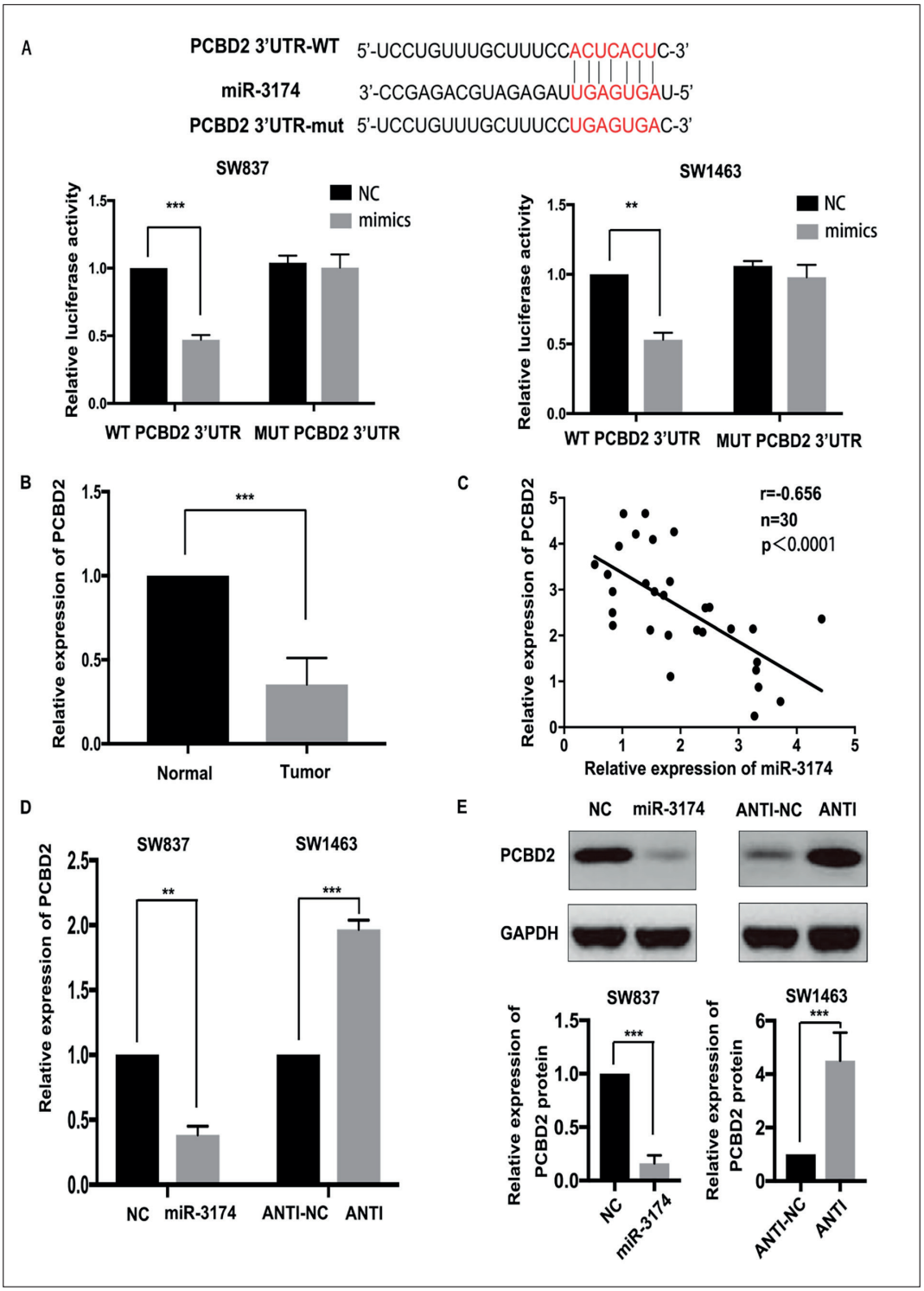

Figure 4. PCBD2 was proved to be the target gene of miR-3174. ***A,*** Luciferase reporter assay was constructed to prove that miR-3174 directly bound to the 3'-UTR of PCBD2. The sequence of wild-type and mutant PCBD2 3'-UTR was shown. After the co-transfection with wild-type or mutant 3'-UTR reporter plasmids, relative PCBD2 luciferase activity was calculated and recorded by histogram. ***B,*** The expression of PCBD2 in rectal cancer tissues and normal tissues. ***C,*** SPSS was used to analysis the correlation between the mRNA level of PCBD2 and the expression of miR-3174 ($p < 0.0001$). ***D,*** PCBD2 was chosen from several databases. QRT-PCR was used to examine the mRNA expression of PCBD2 in transfected cell lines. ***E,*** Western Blot was used to test the protein level of PCBD2 in transfected cell lines. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control group. Data were expressed as mean ± SD.

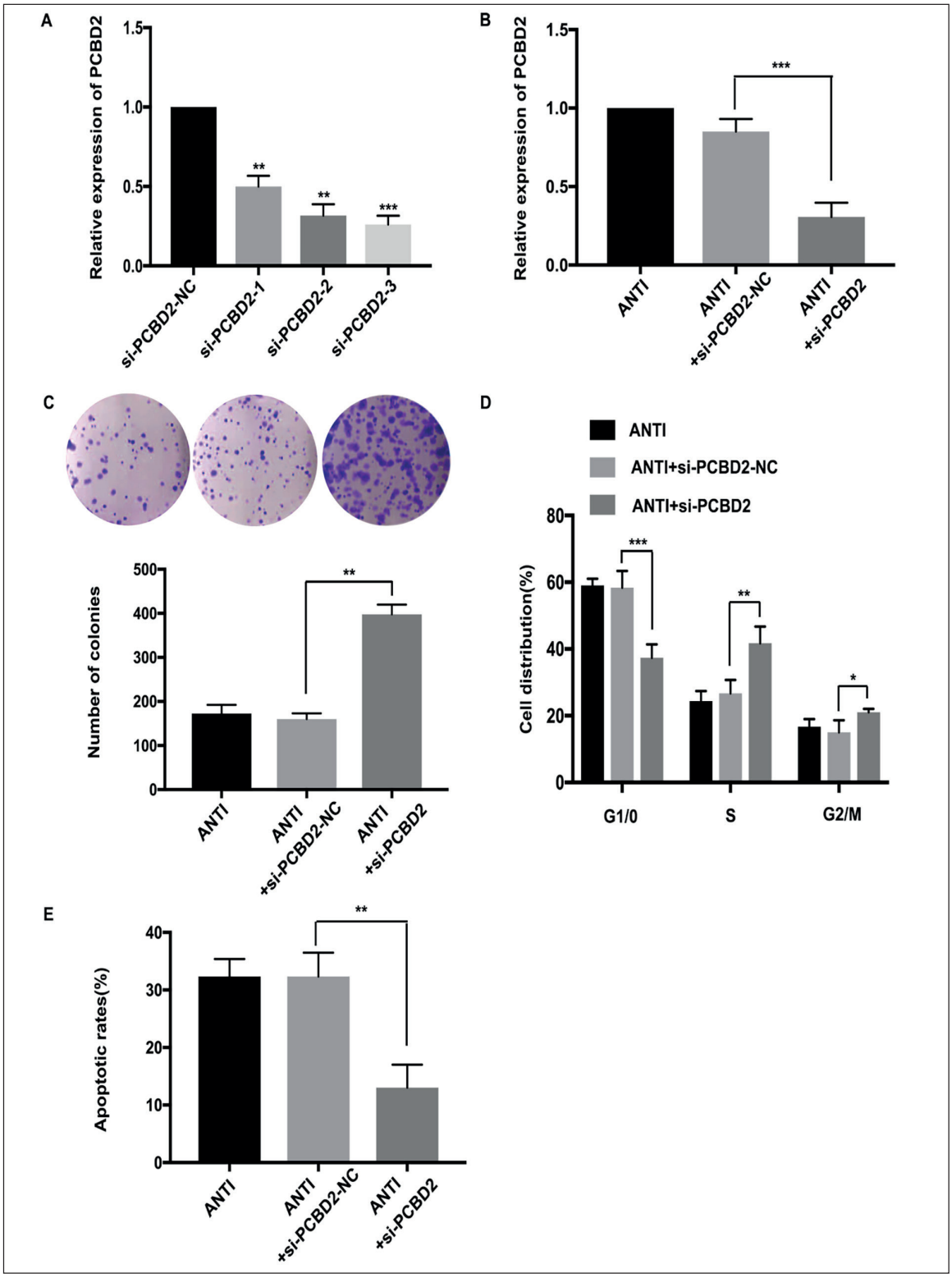

Figure 5. MiR-3174 regulated cell proliferation and viability by directly targeting PCBD2. ***A,*** QRT-PCR was used to examine the mRNA level of PCBD2 in cells transfected with siRNAs. ***B,*** QRT-PCR was used to examine the mRNA level of PCBD2 in cells transfected with si-PCBD2-NC and si-PCBD2. ***C,*** Cell colonies were stained with crystal violet, and the number of colonies was counted. The time for cell colony formation was 10 days. ***D,*** Flow cytometry was used to examine cell cycle. ***E,*** Flow cytometry was used to detect the apoptotic rates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the control group. Data were expressed as mean ± SD.

assay demonstrated that there was no significant changes between the ANTI group and the ANTI+si-PCBD2-NC group. However, colony formation ability of the ANTI+si-PCBD2 group was remarkably decreased. Subsequent flow cytometry analysis indicated that the acceleration effect on cell cycle was abolished when co-transfected with si-PCBD2 (Figure 5D). Besides, we evaluated the apoptotic rate of cells by flow cytometry. Results showed that the promotion influence in cell apoptosis was inhibited when co-transfected with si-PCBD2 (Figure 5E). Hence, we considered that miR-3174 functioned as an oncogene in rectal cancer by regulating PCBD2.

Discussion

About one million people are diagnosed with colorectal cancer each year. It is estimated that 715 thousand deaths are caused by colorectal cancer[12]. Owing to the anatomical differences with colon cancer, local recurrence is a major problem in the treatment of rectal cancer patients. Hence, unlike colon cancer, radiation treatment and different fundamental therapeutics are required[13]. However, the use of chemo-radiotherapy needs specific challenges in the diagnostic and treatment strategies. Therefore, it is vital importance to seek for novel biomarkers and molecular treatment for rectal cancer. MiRNAs have been widely studied in recent years. Studies have reported that miRNAs play specific roles of in the progression of various malignant tumors. For example, miR-22 suppresses the proliferation, migration and invasion of oral cancer by regulating NLRP3[14]. MiR-374b has been proved to function as a tumor suppressor gene in the p38/ERK pathway by targeting JAM-2[15]. MiR-449a is reported to be a potential predictor of colitis-associated colorectal cancer progression[16]. Meanwhile, miR-495 has been proved to enhance the efficacy of radiotherapy in nasopharyngeal cancer by regulating GRP78 and EMT[17]. Furthermore, specific functions of miRNAs have also been reported, including cell proliferation, migration, invasion, apoptosis and differentiation[18-20]. Recently, more and more researchers have given mounting evidence to elucidate the role of miRNAs in various cancers. However, the physiological role of miR-3174 has rarely been elucidated. Herein, in the present study, we firstly used qRT-PCR to examine the expression level of miR-3174 in rectal cancer tissues and normal tissues. Results showed that miR-3174 was significantly up-regulated in rectal cancer tissues and cell lines. Subsequent CCK-8 assay, colony formation assay and flow cytometry indicated that miR-3174 promoted the proliferation while suppressed the apoptosis of rectal cancer cells. In sum, miR-3174 played an oncogenic role in rectal cancer. To our best knowledge, miRNAs usually play as oncogenes or tumor suppressor genes through directly binding to the 3'UTR region of down-stream target genes[11]. To predict the potential target of miR-3174, we searched four public available databases. Result showed that PCBD2 might be an underlying target of miR-3174. Dual luciferase reporter gene assay further validated that PCBD2 was a down-stream target gene of miR-3174. PCBD2 is located at chromosome 5q31.1, coding for a protein involved in tetrahydrobiopterin biosynthesis[21]. However, the role of PCBD2 in rectal cancer has not been well studied. In this research, we indicated that PCBD2 was a direct down-stream target of miR-3174. Meanwhile, PCBD2 was significantly down-regulated in rectal cancer tissues, and was negatively correlated with miR-3174. In addition, rescue experiment suggested that miR-3174 functioned as an oncogene in rectal cancer by targeting PCBD2.

Conclusions

We demonstrated that miR-3174 functioned as an oncogene in rectal cancer by targeting PCBD2, which might bring new insights into the search for novel biomarkers and treatment strategies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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