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MiRNA-875-3p alleviates the progression of colorectal cancer *via* negatively regulating PLK1 level

S.-S. LI[1], H.-J. ZHU[1], J.-Y. LI[2], L.-M. TIAN[1], D.-M. LV[3]

[1]Department of General Surgery, Xi'an Central Hospital, Xi'an, China
[2]Department of Respiratory Medicine Department, Xi'an Central Hospital, Xi'an, China
[3]Department of General Surgery, Yan'an Traditional Chinese Medicine Hospital, Yan'an, China

Shoushuai Li and *Haijun Zhu* contributed equally to this work

Abstract. – OBJECTIVE: To clarify the expression pattern of miRNA-875-3p in CRC and its potential regulatory effect on the progression of CRC.

MATERIALS AND METHODS: MiRNA-875-3p level in 56 matched CRC tissues and adjacent normal tissues were determined. The correlation between the miRNA-875-3p level and pathological indexes of CRC patients was analyzed. Prognostic potential of miRNA-875-3p in CRC patients was assessed by introducing the Kaplan-Meier curves. Influences of miRNA-875-3p on viability, migration, and wound closure were assessed through a series of functional experiments. The interaction between miRNA-875-3p and PLK1 in regulating the progression of CRC was finally uncovered by Dual-Luciferase reporter gene and rescue experiments.

RESULTS: MiRNA-875-3p was downregulated in CRC tissues and cell lines. CRC patients with low level of miRNA-875-3p suffered a higher rate of distant metastasis and worse prognosis. Overexpression of miRNA-875-3p attenuated proliferative and migratory capacities of SW480 and HT29 cells. PLK1 was confirmed to be the target gene of miRNA-875-3p. PLK1 was upregulated in CRC tissues and cell lines, which was negatively regulated by miRNA-875-3p. MiRNA-875-3p alleviated the malignant progression of CRC via negatively regulating PLK1.

CONCLUSIONS: MiRNA-875-3p is downregulated in CRC, which is closely related to distant metastasis and poor prognosis of CRC patients. MiRNA-875-3p alleviates the progression of CRC through targeting and downregulating PLK1.

Key Words:
MiRNA-875-3p, PLK1, CRC, Malignant progression.

Introduction

With the improvement of living standards and changes in the living habits in recent years, the incidence of colorectal cancer (CRC) gradually increases. The morbidity and mortality of CRC rank third and second in malignancies, respectively[1-3]. Surgery is the preferred therapeutic approach for CRC. However, due to the ineffective diagnostic rate at early stage, postoperative recurrence and unsatisfactory outcomes of adjuvant therapies, the overall survival of CRC is relatively poor[4,5]. It is of significance to uncover the mechanism underlying the occurrence and progression of CRC[6,7]. With the advance in multidisciplinary development, the pathogenesis of CRC achieves more understanding[8,9]. The progression of CRC is a complex process involving multiple genes and pathways[10]. Abnormally expressed hallmarks could be utilized as screening methods to improve the therapeutic and prognostic efficacies of CRC[6,8].

MiRNAs are a group of endogenous, short-chain (about 22-26 bases), non-encoding RNAs that are widely found in eukaryotes[11,12]. By binding completely or incompletely to 3'UTR of target genes, miRNAs induce mRNA degradation or translation inhibition to further suppress target gene expressions[10,13]. MiRNAs are widely involved in maintaining cellular behaviors[14]. Several evidence has shown the biological roles of miRNAs in tumor progression, serving as oncogenes or tumor-suppressor genes[14]. In addition, miRNA levels are closely related to tumor staging, therapeutic outcome, and clinical prognosis of tumor diseases, exerting a promising aspect in tumor treatment[15-17].

Corresponding Author: Dongmei Lv, BM; e-mail: 857772640@qq.com

Differentially expressed miRNAs in CRC have been discovered by analyzing microarrays[18]. MiRNA-875-3p is abnormally expressed in CRC[18]. To identify the biological function of miRNA-875-3p in the progression of CRC, searching for the target genes of miRNA-875-3p through bioinformatics method is necessary[13]. One of the potential targets is PLK1, a tumor-related gene involved in many types of tumors. In this paper, we mainly investigated the regulatory effects of miRNA-875-3p/PLK1 on the progression of CRC.

Patients and Methods

Patients and Tissue Samples

56 CRC patients undergoing rectal surgery in Xi'An Central Hospital were enrolled in this investigation. CRC tissues and matched adjacent normal tissues (5 cm away from tumor edge) were surgically resected and preserved within 5 min *ex vivo*. Clinical indexes and follow-up data of CRC patients were collected for further analyses. Patients and their families have been fully informed. This research was approved by the Ethics Committee of Xi'An Central Hospital, and it was conducted in accordance with the Declaration of Helsinki.

Cell Culture

Colon epithelial cell line FHC and CRC cell lines DLD-1, HCT-8, HCT-116, SW480, and HT29 were purchased from ATCC (Manassas, VA, USA). SW480, DLD-1, and HT29 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo-Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), while the others were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640). Until 80-90% confluence, the cell passage was performed using 1×trypsin + ethylene diamine tetraacetic acid (EDTA).

Transfection

Transfection plasmids were provided by GenePharma, Shanghai. Cells seeded in the 6-well plates were transfected using Lipofectamine 3000 at 70% of confluence. At 48 h, cells were harvested for verification of transfection efficacy and subsequent investigations.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in the 96-well plate with 2×10^3 cells per well. At 6, 24, 48, and 72 h, the absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Migration Assay

Cells were adjusted to a dose of 2.0×10^5/mL. 200 µL suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA) and inserted in a 24-well plate. In the bottom side, 700 µL of medium containing 10% FBS was applied. After 48 h of incubation, cells migrated to the bottom side were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. The number of migratory cells was counted in 5 randomly selected fields per sample (magnification 200×).

Wound Healing Assay

Cells were seeded in a 24-well plate with 5.0×10^5 cells/well. After cell adherence (0 h), an artificial wound was created in the confluent cell monolayer using a 200 µL pipette tip. Wound closure images were taken at 0 and 24 h using an inverted microscope, respectively. Percentage of wound closure was calculated.

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

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), purified by DNase I treatment, and reversely transcribed into cDNA using Primescript RT Reagent (Takara, Otsu, Shiga, Japan). The 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 references. Each sample was performed in triplicate, and the relative level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer 5.0 was used to design the qRT-PCR primers.

Dual-Luciferase Reporter Gene Assay

SW480 and HT29 cells co-transfected with miRNA-875-3p-WT/miRNA-875-3p-MUT and NC/pcDNA-PLK1, respectively. After co-transfection for 48 h, cells were lysed for determining the luciferase activity.

Wound Healing Assay

Cells were seeded in a 24-well plate with 5.0×10^5 cells/well. After cell adherence (0 h), an artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. Wound closure images were taken at 0 and 24 h using an inverted microscope, respectively. The percentage of wound closure was calculated.

Statistical Analysis

GraphPad Prism 5 V5.01 (Version X; La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. Kaplan-Meier curves were introduced for survival analysis. Chi-square test was performed to evaluate the correlation between miRNA-875-3p level with clinical indexes of CRC patients. $p < 0.05$ was considered as statistically significant.

Results

MiRNA-875-3p was Downregulated in CRC

A total of 56 matched CRC tissues and adjacent normal tissues were collected. QRT-PCR data showed downregulated miRNA-875-3p in CRC tissues relative to the normal ones (Figure 1A). Identically, the miRNA-875-3p level remained lower in CRC compared to that of the colon epithelial cell line (Figure 1B). Among the five selected CRC cell lines, SW480 and HT29 cells expressed the lowest abundance of miRNA-875-3p, which were utilized for the subsequent investigations.

MiRNA-875-3p was Correlated with Distant Metastasis and Overall Survival in CRC Patients

Based on the median level of miRNA-875-3p in the enrolled 56 CRC patients, they were assigned into high-level and low-level miRNA-875-3p group. By analyzing their pathological indexes, miRNA-875-3p level was found to be negatively correlated with distant metastasis, rather than age, gender, TNM staging, and lymphatic metastasis of CRC patients (Table I). Moreover, the Kaplan-Meier curves demonstrated that CRC patients in low-level group experienced a worse survival (Figure 1C).

Overexpression of MiRNA-875-3p Suppressed Proliferative and Migratory Capacities of CRC

MiRNA-875-3p mimics were constructed to help to further uncover the biological function of miRNA-875-3p. Transfection of miRNA-875-3p mimics remarkably elevated the miRNA-875-3p level in SW480 and HT29 cells (Figure 2A). In CRC cells overexpressing miRNA-875-3p, viabilities of SW480 and HT29 cells were markedly

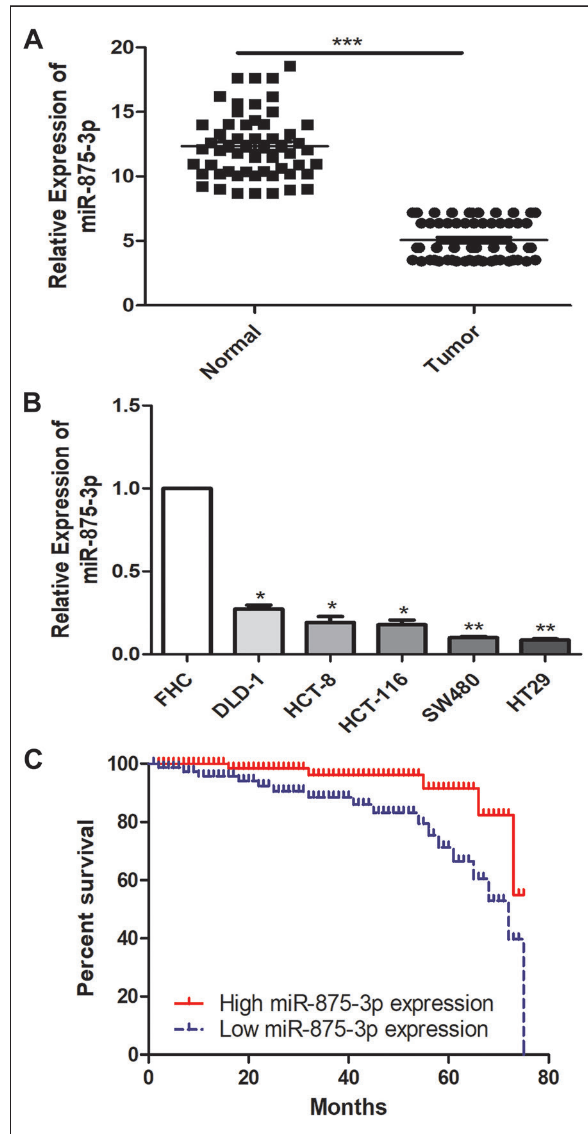

Figure 1. MiR-875-3p was downregulated in CRC. **A,** MiR-875-3p level in CRC tissues and adjacent normal tissues. **B,** MiR-875-3p level in colon epithelial cell line FHC and CRC cell lines DLD-1, HCT-8, HCT-116, SW480, and HT29. **C,** Kaplan-Meier curves revealed overall survival in CRC patients expressing high or low level of miR-875-3p.

Table I. Association of miR-875-3p and PLK1 expression with clinicopathologic characteristics of colorectal cancer.

Parameters	No. of cases	MiR-875-3p expression		*p*-value	PLK1 expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.174			0.503
< 60	23	14	9		8	15	
≥ 60	33	14	19		15	18	
Gender				0.285			0.415
Male	28	16	12		10	18	
Female	28	12	16		13	15	
T stage				0.057			0.39
T1-T2	33	20	13		12	21	
T3-T4	23	8	15		11	12	
Lymph node metastasis				0.094			0.311
No	36	21	15		13	23	
Yes	20	7	13		10	10	
Distance metastasis				**0.014**			**0.012**
No	33	21	12		9	24	
Yes	23	7	16		14	9	

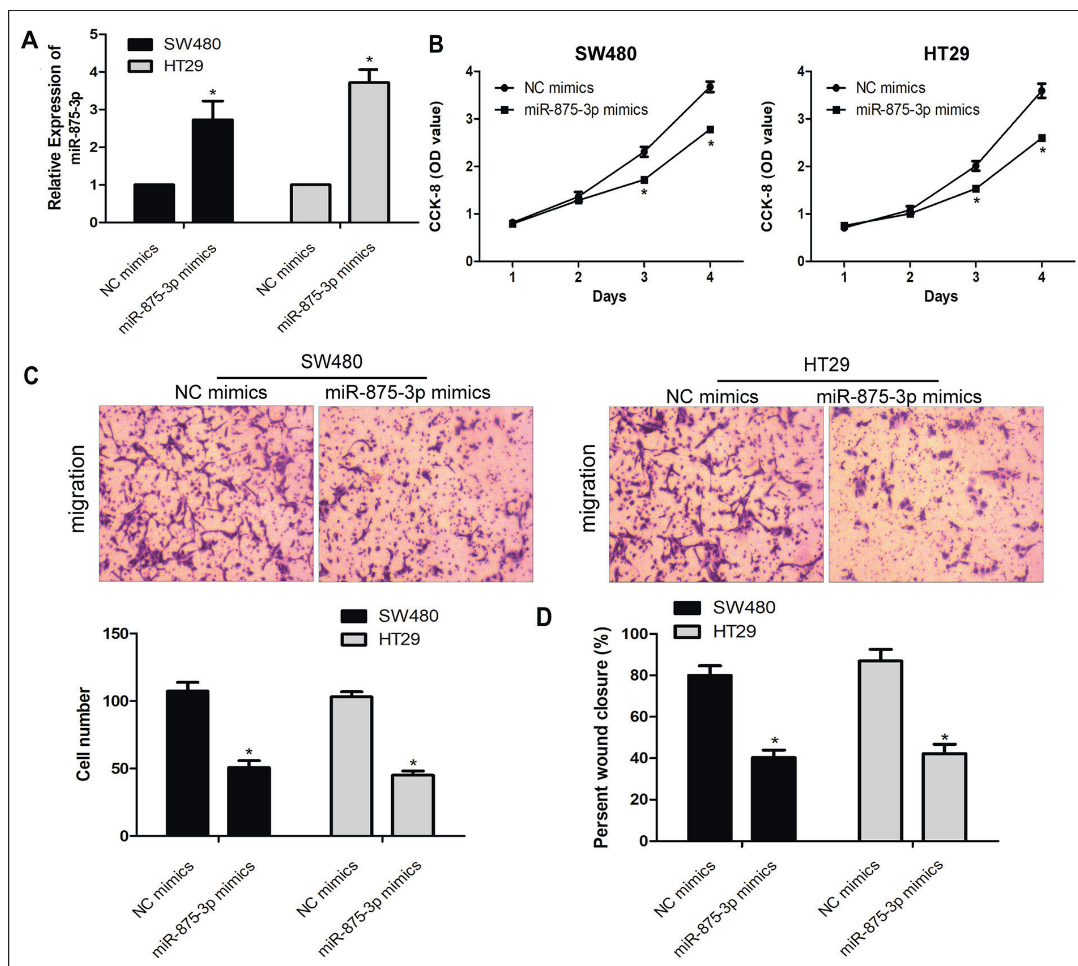

Figure 2. Overexpression of miR-875-3p suppressed proliferative and migratory capacities of CRC. **A,** Transfection efficacy of miR-875-3p mimics in SW480 and HT29 cells. **B,** Viability in SW480 and HT29 cells transfected with NC mimics or miR-875-3p mimics at day 1, 2, 3, and 4. **C,** Migration in SW480 and HT29 cells transfected with NC mimics or miR-875-3p mimics (magnification 200×). **D,** Wound closure in SW480 and HT29 cells transfected with NC mimics or miR-875-3p mimics.

reduced at day 3 and 4 (Figure 2B). Migratory capacity decreased after overexpression of miRNA-875-3p in CRC cells (Figure 2C). Additionally, the percentage of wound closure declined after the transfection of miRNA-875-3p mimics in CRC cells (Figure 2D).

Interaction Between MiRNA-875-3p and PLK1

Potential binding sites were discovered in the promoter regions of miRNA-875-3p and PLK1 (Figure 3A). SW480 and HT29 cells were co-transfected with miRNA-875-3p-WT/miRNA-875-3p-MUT and NC/pcDNA-PLK1, respectively. As the data showed, the overexpression of PLK1 markedly reduced the luciferase activity in miRNA-875-3p-WT vector, while no significant difference was found in the miRNA-875-3p-MUT vector (Figure 3B). We verified that a binding relation existed between miRNA-875-3p and PLK1. In CRC cells overexpressing miRNA-875-3p, the relative level of PLK1 was markedly downregulated (Figure 3C). Furthermore, PLK1 was upregulated in CRC tissues and cell lines (Figure 3D, 3E). A negative correlation was identified between the expression levels of miRNA-875-3p and PLK1 in CRC tissues (Figure 3F).

MiRNA-875-3p Influenced the Progression of CRC Via Negatively Regulating PLK1 Expression

To further uncover the interaction between miRNA-875-3p and PLK1 in influencing the progression of CRC, a series of rescue investigations were conducted. Transfection of miRNA-875-3p mimics markedly upregulated PLK1 level in SW480 and HT29 cells. Also, it was downregulated by the co-transfection of pcDNA-PLK1 (Figure 4A). Notably, the overexpression of PLK1 reversed the attenuated proliferative and migratory abilities in CRC cells overexpressing miRNA-875-3p (Figure 4B-4D).

Discussion

CRC is the prevalent cancer in clinical practice. The lack of sufficient diagnostic approach at early stage and the high rate of postoperative recurrence seriously restrict the therapeutic efficacy of CRC[8-10]. Therefore, it is urgent to uncover the mechanism underlying the metastasis and recurrence of CRC. Previous studies[10,11] have demonstrated the crucial importance of miRNAs in the occurrence and progression of tumors. Differentially expressed miRNAs and lncRNAs exert their potentials in diagnosing, treating, and prognosing tumor diseases as a non-invasive method[9-14].

MiRNA-875-3p is found to be lowly expressed in many types of tumors, and its level may be related to tumor prognosis[19]. Our findings uncovered that miRNA-875-3p was downregulated in CRC tissues and cell lines, suggesting the potential role of miRNA-875-3p as a tumor suppressor in CRC. The survival analysis yielded a conclusion that CRC patients expressing a low level of miRNA-875-3p suffered a higher rate of distant metastasis and worse prognosis relative to those expressing a high level of miRNA-875-3p. In SW480 and HT29 cells, the overexpression of miRNA-875-3p attenuated their proliferative and migratory capacities, further revealing the tumor-suppressor effect of miRNA-875-3p on CRC.

By complete or incomplete binding to target sites (coding region or open reading frame of mRNA), miRNAs induce mRNA degradation, which is frequently seen in plants[13,14]. A single miRNA could have multiple target genes, and several miRNAs could target the same gene. Such a complex regulatory network precisely mediates gene expressions[10,13]. The previous work has predicted the binding sites in the promoter regions of miRNA-875-3p and PLK1. In this paper, the Dual-Luciferase reporter gene assay showed that PLK1 was the target gene of miRNA-875-3p. PLK1 was upregulated in CRC tissues and cell lines. Besides, a negative correlation was identified between expression levels of miRNA-875-3p and PLK1 in CRC tissues. As a result, we speculated whether PLK1 was involved in the progression of CRC influenced by miRNA-875-3p. Rescue experiments revealed that silence of PLK1 reversed the accelerated proliferative and migratory capacities in SW480 and HT29 cells overexpressing miRNA-875-3p. Hence, a positive feedback loop miRNA-875-3p/PLK1 was identified, which attenuated the malignant progression of CRC.

Conclusions

This study disclosed that miRNA-875-3p was downregulated in CRC, which was closely related to distant metastasis and poor prognosis of CRC patients. MiRNA-875-3p alleviated the progression of CRC through targeting and downregulating PLK1.

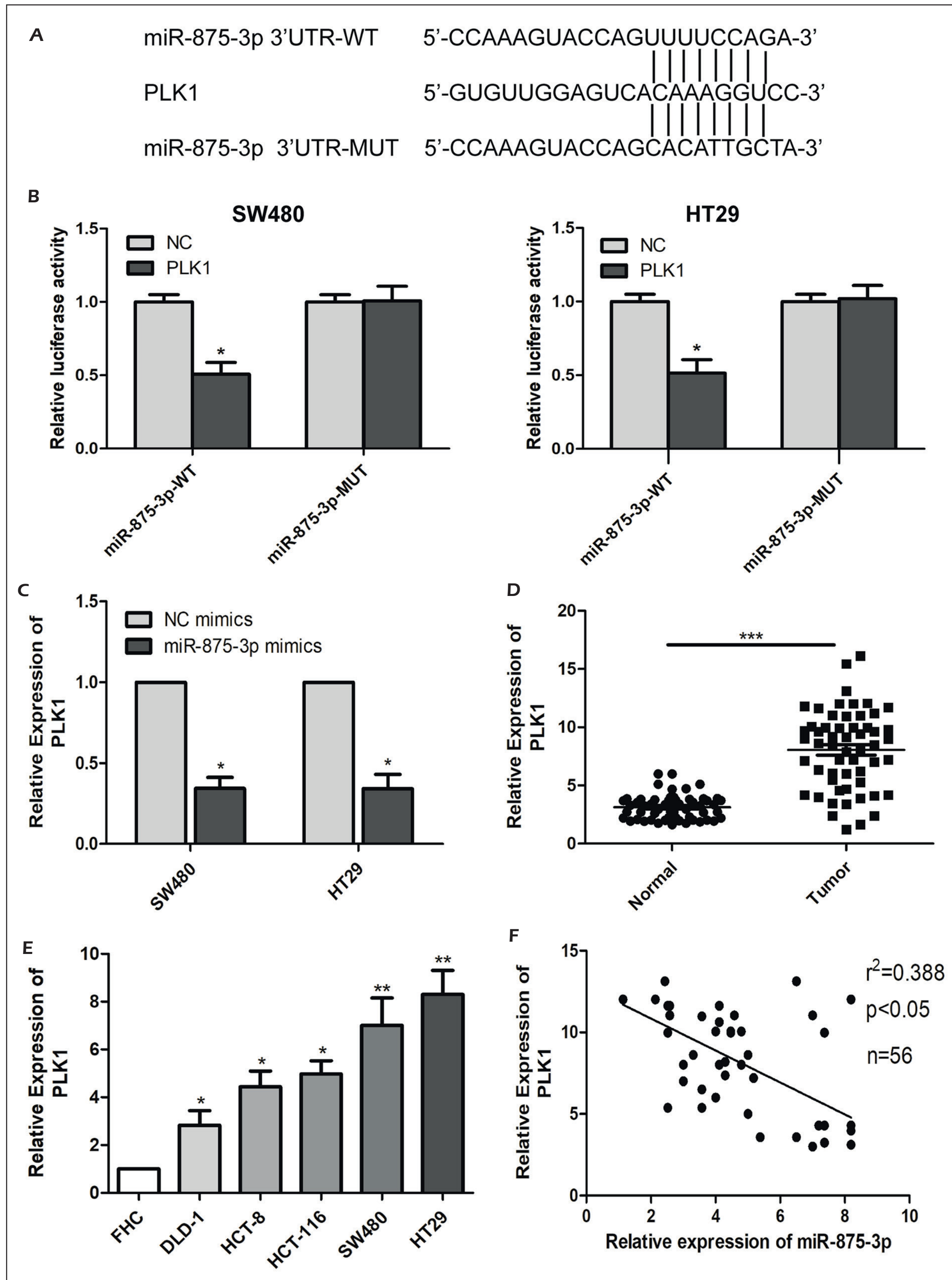

Figure 3. Interaction between miR-875-3p and PLK1. **A,** Binding sites in the promoter regions of miR-875-3p and PLK1. **B,** Luciferase activity in SW480 and HT29 cells co-transfected with miR-875-3p-WT/miR-875-3p-MUT and NC/pcDNA-PLK1, respectively. **C,** PLK1 level in SW480 and HT29 cells transfected with NC mimics or miR-875-3p mimics. **D,** PLK1 level in CRC tissues and adjacent normal tissues. **E,** PLK1 level in colon epithelial cell line FHC and CRC cell lines DLD-1, HCT-8, HCT-116, SW480, and HT29. **F,** Negative correlation between expression levels of miR-875-3p and PLK1 in CRC tissues.

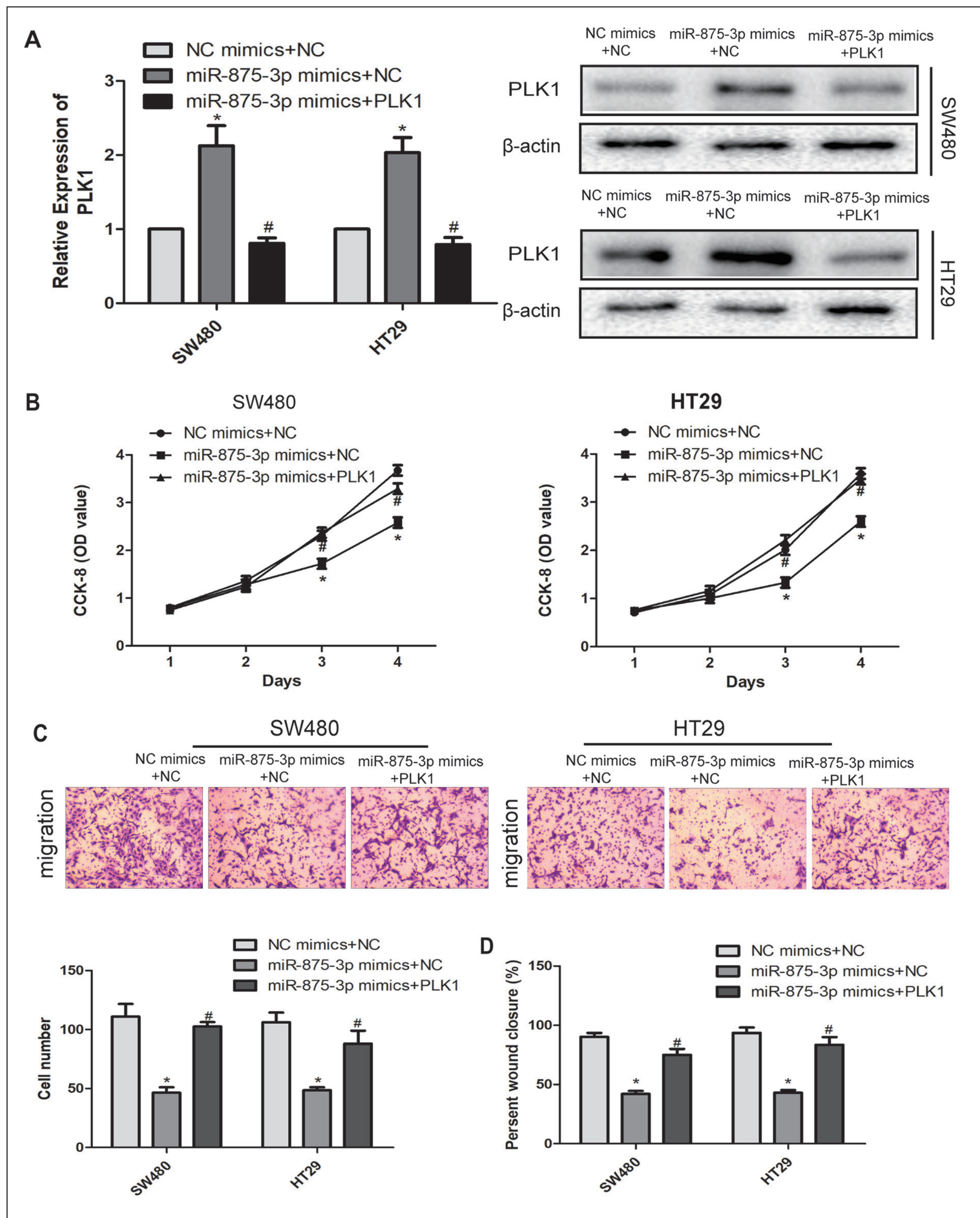

Figure 4. MiR-875-3p influenced the progression of CRC *via* negatively regulating PLK1 expression. SW480 and HT29 cells were transfected with NC mimics + NC, miR-875-3p mimics + NC or miR-875-3p mimics + pcDNA-PLK1. **A,** mRNA and protein levels of PLK1; **B,** Viability at day 1, 2, 3, and 4; **C,** Migratory cell number (magnification: 40×); **D,** Wound closure.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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