

MiR-9-5p suppresses cell metastasis and epithelial-mesenchymal transition through targeting FOXP2 and predicts prognosis of colorectal carcinoma

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Abstract. – **OBJECTIVE:** Colorectal carcinoma (CRC) is a common malignant tumor of the digestive tract that occurs in the colon, and the incidence is the third in the gastrointestinal tumor. Recently, the dysregulated expression of microRNA-9 (miR-9) has been identified in many human cancers. However, the special function of miR-9 in the progression of colorectal carcinoma (CRC) remains unknown.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of miR-9-5p in 72 pairs of CRC tissues and cell lines. The correlation between miR-9-5p expression and clinical features or prognosis of CRC patients was analyzed. In addition, we examined the mRNA and protein expression levels of forkhead box P2 (FOXP2) using Western blot analysis and qRT-PCR. The functions of miR-9-5p and FOXP2 were investigated using transwell assay and epithelial-mesenchymal transition (EMT). The relation between miR-9-5p and FOXP2 was confirmed by the dual-luciferase assay.

RESULTS: In this study, down-regulation of miR-9-5p and up-regulation of the forkhead box P2 (FOXP2) were detected in CRC tissues and cell lines. Moreover, miR-9-5p was found to inhibit cell metastasis and EMT in CRC. In addition, it was confirmed that miR-9-5p directly targeted FOXP2 in CRC. Furthermore, FOXP2 had a carcinogenic effect on CRC. And the over-expression of FOXP2 weakened the suppressive effect of miR-9-5p in CRC. Of note, we observed found that the low expression of miR-9-5p and the high expression of FOXP2 were correlated with poor prognosis of CRC patients.

CONCLUSIONS: MiR-9-5p suppressed cell metastasis and EMT through targeting FOXP2. Furthermore, dysregulation of miR-9-5p predicted the prognosis of CRC patients. Therefore, miR-9-5p may be a biomarker for cell metastasis and a prognostic factor for CRC patients. MiR-9-5p/FOXP2 axis will provide a new breakthrough in the diagnosis and treatment of CRC.

Key Words:

Colorectal carcinoma, MiR-9-5p, Epithelial-to-mesenchymal transition, Metastasis, Prognosis, FOXP2.

Introduction

Colorectal carcinoma (CRC) is a common malignant tumor in the digestive tract, second only to gastric cancer and esophageal cancer¹. In the past two decades, the incidence of CRC has gradually increased². In particular, liver metastasis is the main cause of death in CRC patients. How to improve the efficacy of liver metastasis has become one of the focuses in the field of CRC research³. Moreover, liver metastasis is an important factor affecting the prognosis of CRC patients⁴. Epithelial-to-mesenchymal transition (EMT) associated with tumor recurrence plays an important role in tumor metastasis⁵. However, the regulatory mechanism of EMT and tumor metastasis is complex in CRC and needs to be further explored.

EMT is a transformation process by which epithelial cells transform into mesenchymal cells, conferring the ability to metastasize and invade cells⁶. EMT is also an important step in cancers and other pathological processes involving organ regeneration, such as organ fibrosis⁷. In the development of tumors, the down-regulation of E-cadherin is considered a key step⁸. Previous studies have shown that some genes and signaling pathways can regulate EMT in CRC, such as Trx-1, S100P⁹, DCLK1¹⁰, and Wnt/ β -catenin/Snail signaling pathways¹¹. Moreover, there are complex regulatory mechanisms of miRNAs during the EMT process.

MicroRNAs (miRNAs) can affect corresponding gene expression at the post-transcriptional level by specifically binding to mRNA. It is estimated

that miRNAs affect 30% of protein expression in human¹². During tumor development, miRNAs can function as proto-oncogenes and tumor suppressor genes by up-regulating and down-regulating themselves¹³. For instance, miR-522¹⁴, miR-124¹⁵, miR-132¹⁶, miR-494¹⁷, and miR-29c¹⁸ have been identified to regulate the development and progression of CRC. In the present work, we aimed to achieve the function of miR-9-5p in human CRC through regulating the forkhead box P2 (FOXP2). It was found that miR-9 expression was decreased in the peripheral leukocytes of Huntington's disease¹⁹. Furthermore, miR-9 was found to act as a biomarker for the diagnosis and prognosis of the esophageal squamous cell carcinoma²⁰. These researches indicated that miR-9 was expressed abnormally in human cancer and could regulate tumorigenesis.

In this study, the expression of miR-9-5p was examined in CRC cell lines and tissues. The effects of miR-9-5p on tumor metastasis and EMT were analyzed. Furthermore, the interaction between miR-9-5p and FOXP2 was explored CRC. Meanwhile, we also examined the relation between miR-9-5p and FOXP2 or the prognosis of CRC patients.

Patients and Methods

Clinical Tissues

Seventy-two pairs of surgical CRC specimens and adjacent tissues were obtained from the Liaocheng People's Hospital. All of these CRC patients did not receive any treatment prior to surgery. These tissues were then frozen in liquid nitrogen and stored in a -80°C refrigerator for further experiment. Written informed consents were obtained from all participants prior to the study. This investigation was approved by the Ethics Committee of Liaocheng People's Hospital.

Cell Culture and Transfection

HT29, HCT116, SW480, SW620, and HEK293T cell lines were used for this investigation. These cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All of these cell lines were seeded in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) with 10% of fetal bovine serum (FBS; Gibco, Rockville, MD, USA). They were cultured in an incubator at 37°C with 5% of CO_2 .

The miR-9-5p mimic or inhibitor and FOXP2 siRNA (si-FOXP2) were purchased from GenePharma (Shanghai, China). They were then severally transferred to CRC cells with Lipofectamine

2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturers' protocols.

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

Total RNA containing miRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to quantify the expression of miR-9-5p in CRC tissues and cell lines. Quantitative RT-PCR was performed on ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) by the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for miR-9-5p and FOXP2. Finally, their expressions were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. The primers used in this study are listed as follows: miR-9-forward: 5'-GCTG-GAGAACCCGTAGATCCGAT-3'; miR-9-reverse: 5'-GTGCAGGGTCCGAGG T-3'; U6-forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3'; U6-reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'; FOXP2-forward: 5'-TGTGT-CATAGGTTAGGTGGTGA -3'; FOXP2-reverse: 5'-AGCCAATTCTTTTGTGTTCGTG-3'; GAPDH-forward: 5'-AGAAGGCTGGGGCT-CATTTG-3'; GAPDH-reverse: 5'-AGGGGC-CATCCACAGTCTTC-3'.

Dual Luciferase Assay

The 3'-Untranslated Region (3'-UTR) of wild or mutant type FOXP2 was inserted into the pmir-GLO luciferase vector (Promega, Madison, WI, USA) for luciferase reporter experiments. Next, the luciferase vector and miR-9-5p mimics were transfected into HEK293T cells. After 48 h, the dual-luciferase assay system (Promega, Madison, WI, USA) was used to analyze the luciferase activity.

Transwell Assays for Cell Migration and Invasion

Cell migration and invasion were assessed using transwell chambers (8 μm pore size; Millipore, Billerica, MA, USA). First, 1×10^5 CRC cells without serum were placed in the upper chamber on the uncoated membrane. The lower chamber was filled with 10% of FBS to induce CRC cell migration or invasion. Cells were then placed in the upper chamber along with the coated membrane for the invasion assay. Next, these cells were incubated for cell migration and invasion. After 24 h, the cells were stained with 0.1% of crystal violet. A microscope was used to count migrated and invading cells.

Western Blot Analysis

Protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein was then separated by 10% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the protein was transferred in polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and incubated with 5% of non-fat milk at room temperature. After that, we incubated the membrane with rabbit monoclonal anti-FOXP2 (1:2000; Abcam, Cambridge, MA, USA), rabbit monoclonal anti-GAPDH antibody (1:1000; Epitomics, Burlingame, CA, USA) overnight at 4°C. The protein was incubated with goat polyclonal anti-rabbit IgG secondary antibody (1:2000; Abcam, Cambridge, MA, USA). Finally, protein expression levels were measured by enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA). In addition, antibodies against Vimentin, E-cadherin, and N-cadherin were obtained from Abcam (Cambridge, MA, USA).

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Armonk, NY, USA) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). The difference was calculated according to the Chi-squared test. The relation between miR-9-5p expression and survival rate was performed by the Kaplan-Meier method and the log-rank test. $p < 0.05$ was defined as a significant difference.

Results

The Expressions of MiR-9-5p and FOXP2 were Detected in CRC

Primarily, mRNA expressions of miR-9-5p and FOXP2 were measured in CRC. Results showed that miR-9-5p was down-regulated in CRC tissues and cell lines compared to the control (Figure 1A, 1B). In contrast, FOXP2 expres-

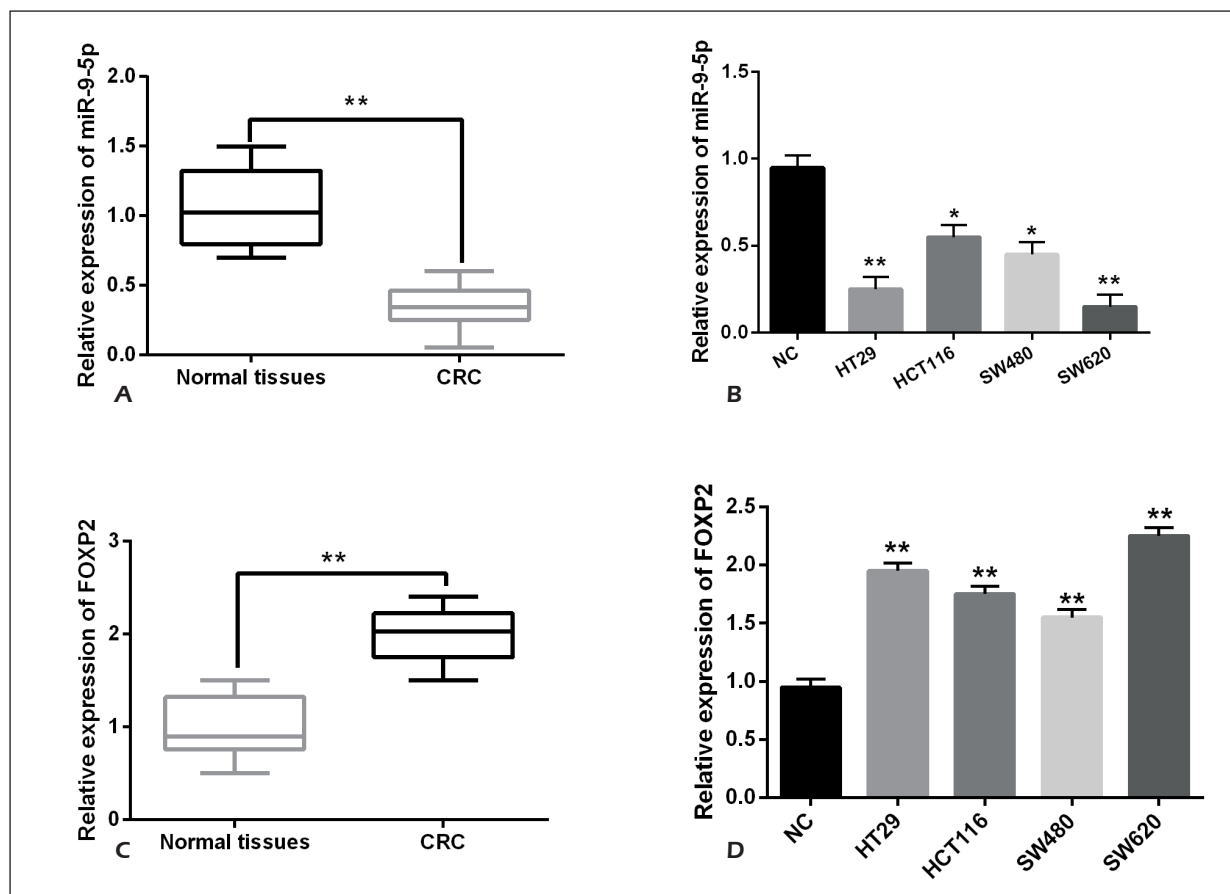


Figure 1. Expressions of miR-9-5p and FOXP2 detected in CRC. **A**, Expressions of miR-9-5p in CRC tissues detected via qRT-PCR. **B**, Expression of miR-9-5p in HT29, HCT116, SW480, and SW620 cells. **C**, Expressions of FOXP2 in CRC tissues detected via qRT-PCR. **D**, Expression of FOXP2 in HT29, HCT116, SW480, and SW620 cells. * $p < 0.05$, ** $p < 0.01$.

Table I. Correlation between the clinicopathologic characteristics and miR-9-5p and FOXP2 expression in CRC.

Characteristics	Number of cases (n=72)	miR-9-5p		p-value	FOXP2		p-value
		High	Low		High	Low	
Age (years)				0.372			0.421
≥ 60	34	14	20		18	16	
< 60	38	13	25		23	15	
Gender				0.573			0.469
Male	37	17	20		22	15	
Female	35	13	22		18	17	
Tumor size (cm)				0.72			0.643
≥ 5	40	16	24		25	15	
< 5	32	16	16		17	15	
TNM stage				0.014*			0.003**
I + II	52	20	32		29	23	
III + IV	20	8	12		11	9	
Lymph node				0.026*			0.018*
No metastasis	49	18	31		29	20	
Metastasis	23	8	15		14	9	

Statistical analyses were performed by the χ^2 -test. TNM, tumor-node-metastasis. * $p < 0.05$ was considered significant.

sion was higher in CRC tissues and cell lines than that in the control (Figure 1C, 1D). We then investigated the correlation between clinicopathological characteristics and miR-9-5p or FOXP2 in CRC. Results suggested that low expression of miR-9-5p was correlated with tumor-node-metastasis stage (TNM, $p=0.014$) and lymph node metastasis ($p=0.026$), while high FOXP2 expression was associated with TNM stage ($p=0.003$) and lymph node metastasis ($p=0.018$) as well (Table I). These results suggested that aberrant expressions of miR-9-5p and FOXP2 may be related to metastasis and prognosis of CRC.

MiR-9-5p Inhibited Cell Metastasis and EMT in CRC

MiR-9-5p mimics or inhibitor was transfected into SW620 cells to further investigate its role in CRC. Next, the expression levels of miR-9-5p were measured in transfected cells *via* qRT-PCR (Figure 2A). In addition, expressions of three markers (epithelial marker E-cadherin, mesenchymal marker N-cadherin, and Vimentin) in EMT were identified by the Western blot analysis. Notably, the high expression of miR-9-5p promoted E-cadherin expression and decreased N-cadherin and Vimentin expressions. Correspondingly, the opposite result was found in SW620 cells with miR-9-5p inhibitor (Figure 2B). Therefore, we suspect that miR-9-5p has the same propensity for cell metastasis in CRC. The abilities of cell migration and invasion were then identified in these trans-

fected cells. As we expected, the miR-9-5p mimic significantly inhibited cell migration, while the cell miR-9-5p inhibitor promoted migration in SW620 cells (Figure 2C). The same tendency of cell invasion was found in SW620 cells with miR-9-5p mimics or inhibitor (Figure 2D). Briefly, miR-9-5p inhibited cell metastasis and EMT in CRC.

MiR-9-5p Directly Targeted FOXP2 in CRC

The TargetScan database (<http://www.targetscan.org/>) showed that FOXP2 was a target gene of miR-9-5p (Figure 3A). We, then, performed a dual-luciferase reporter assay to verify the relation between miR-9-5p and FOXP2. Results showed that miR-9-5p mimics blocked the luciferase activity of wild-type FOXP2. However, miR-9-5p mimics had no effect on the mutant type FOXP2 (Figure 3B). Additionally, we also found a negative association between miR-9-5p and FOXP2 expressions in CRC tissues ($R^2=0.475$, $p<0.01$, Figure 3C). Besides that, we examined the mRNA and protein expression levels of FOXP2 in SW620 cells with miR-9-5p mimics or inhibitor. Results suggested that the overexpression of miR-9-5p significantly suppressed FOXP2 expression. At the same time, the down-regulation of miR-9-5p promoted FOXP2 expression in SW620 cells (Figure 3D and 3E). Therefore, it was found that miR-9-5p directly targeted FOXP2 and was negatively correlated with FOXP2 in CRC.

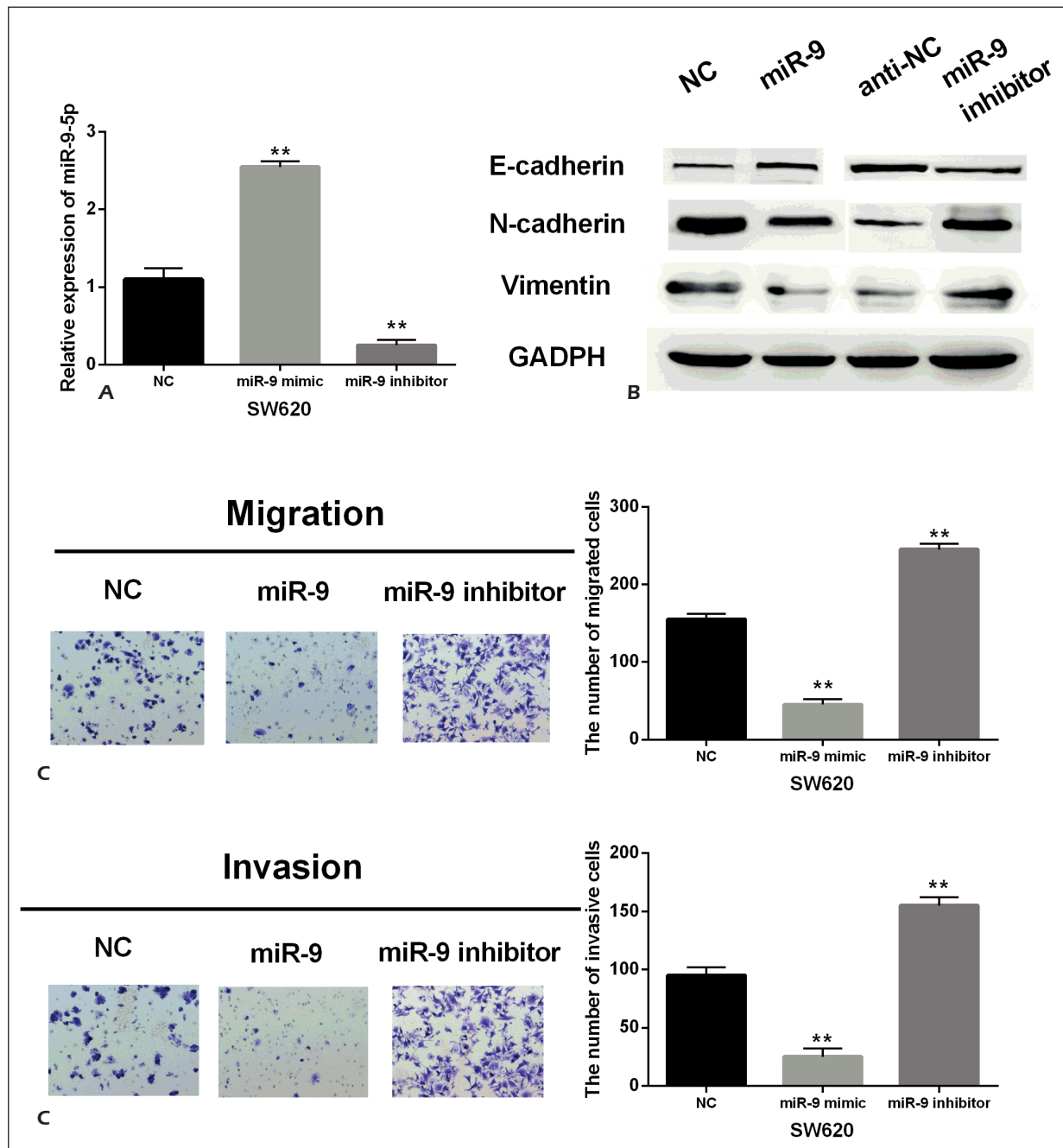


Figure 2. MiR-9-5p inhibited cell metastasis and EMT in CRC. **A**, Expression of miR-9-5p was examined in SW620 cells with miR-9-5p mimics or inhibitor. **B**, Western blot analysis of E-cadherin, N-cadherin, and Vimentin in SW620 cells with miR-9-5p mimics or inhibitor. **C-D**, Cell migration and invasion were measured in cells with miR-9-5p mimics or inhibitor using transwell analysis (magnification: 40×). ** $p<0.01$.

FOXP2 Functioned as an Oncogene in CRC

FOXP2 siRNA was transfected into SW620 cells to investigate its effect on cell metastasis and EMT in CRC. The expression level of FOXP2 was detected by qRT-PCR in transfected cells (Figure 4A). Regarding EMT, the knockdown of FOXP2

promoted E-cadherin expression and decreased N-cadherin and Vimentin expressions (Figure 4B). Besides that, we also found that FOXP2 silencing suppressed migration and invasion of SW620 cells (Figure 4C and 4D). In summary, the knockdown of FOXP2 showed an inhibitory effect on cell metastasis and EMT in CRC.

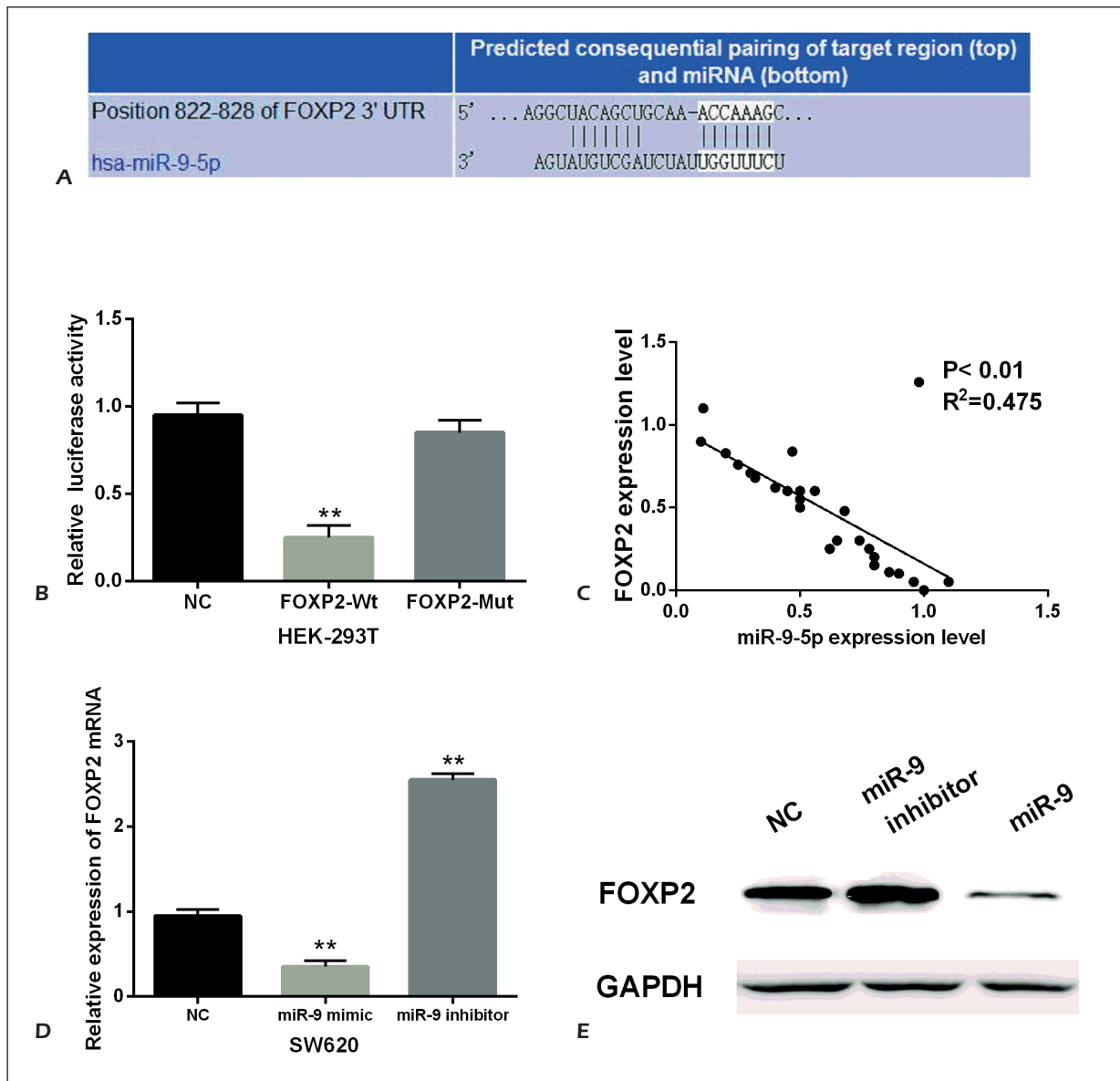


Figure 3. MiR-9-5p directly targeted FOXP2 in CRC. **A**, Binding sites of miR-9-5p on the 3'-UTR of FOXP2. **B**, Luciferase reporter assay. **C**, Correlation between miR-9-5p and FOXP2. **D-E**, The mRNA and protein expressions of FOXP2 were analyzed in cells with miR-9-5p mimics or inhibitor. $**p < 0.01$.

Overexpression of FOXP2 Weakened the Suppressive Function of MiR-9-5p in CRC

We explored the interaction between miR-9-5p and FOXP2 in SW620 cells. As shown in Figure 5A and 5B, the FOXP2 vector recovered the decreased expression of FOXP2, induced by miR-9-5p mimics in SW620 cells. Functionally, the FOXP2 vector was found to weaken the inhibitory effect of miR-9-5p on migration and invasion of SW620 cells (Figure 5C and 5D). Collectively, upregulation of FOXP2 weakened the inhibitory

effect of miR-9-5p in CRC, indicating that miR-9-5p can inhibit cell metastasis and EMT by modulating FOXP2 expression in CRC.

MiR-9-5p and FOXP2 Could Predict the Prognosis of CRC Patients

Finally, we analyzed the correlation between the survival rate of CRC patients and miR-9-5p or FOXP2 expressions. Kaplan-Meier survival curves suggested that CRC patients with high miR-9-5p expression had a longer overall survival (OS, $p = 0.0135$, Figure 6A) and disease-free

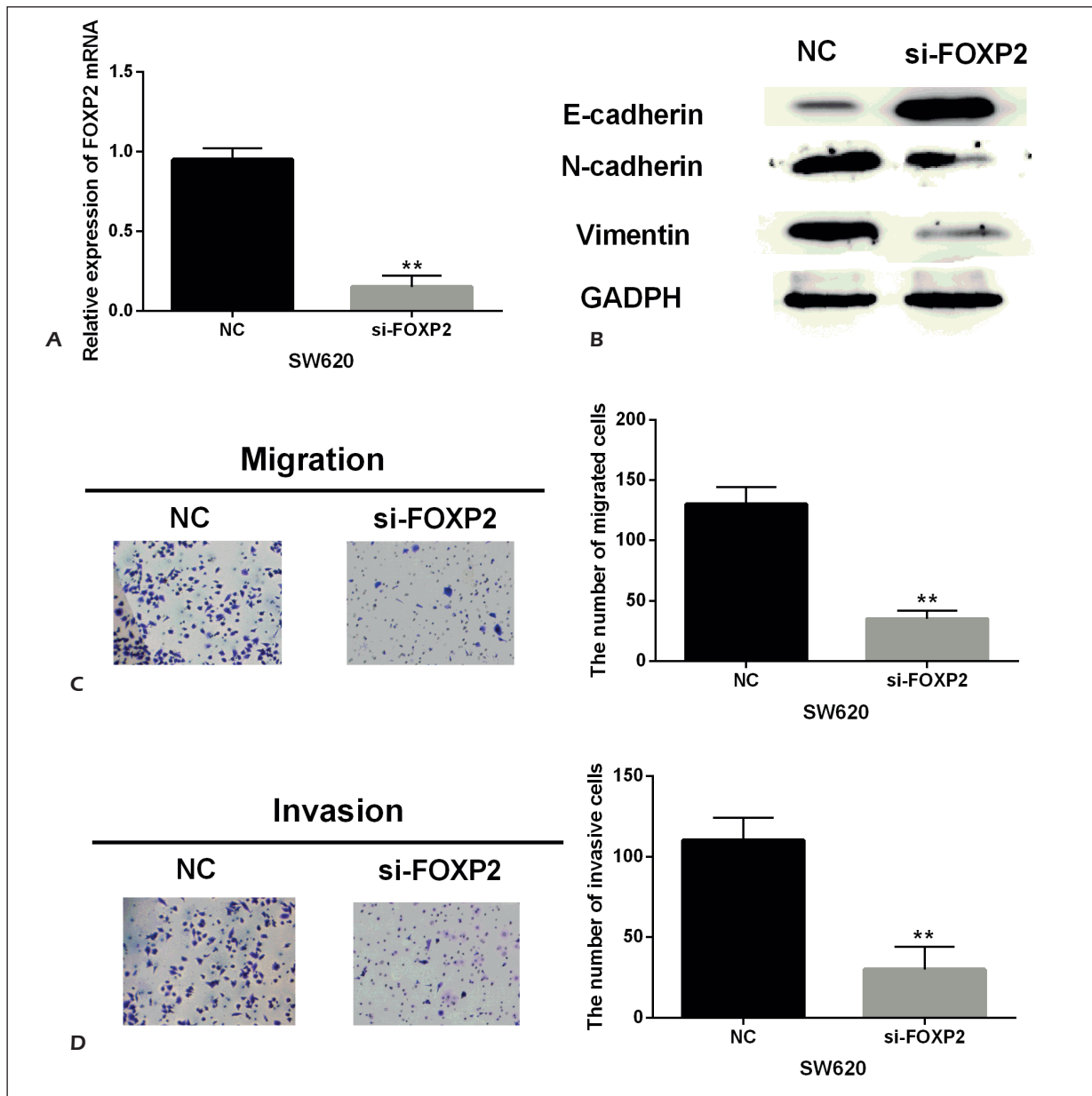


Figure 4. FOXP2 functioned as an oncogene in CRC. **A**, Expression of FOXP2 was measured in SW620 cells with FOXP2 siRNA. **B**, Western blot analysis of E-cadherin, N-cadherin, and Vimentin in SW620 cells with FOXP2 siRNA. **C-D**, Cell migration and invasion analysis of SW620 cells with FOXP2 siRNA (magnification: 40 $\times$). ** p <0.01.

survival (DFS, $p=0.0085$, Figure 6B). Results suggested that low miR-9-5p expression was correlated with a poor prognosis in CRC patients. In contrast, shorter DFS ($p=0.0035$) and OS ($p=0.0019$) were found in CRC patients with high FOXP2 expression (Figure 6C and 6D). It indicated that the high expression of FOXP2 was related to a poor prognosis of CRC patients. Briefly, low expression of miR-9-5p and high expression of FOXP2 predicted poor prognosis in CRC patients.

Discussion

In recent years, aberrantly expressed miRNAs have been found to be associated with a variety of biological processes in many human cancers. In particular, dysregulation of miR-9 had been identified in hepatocellular carcinoma²¹, epithelial ovarian cancer²², medullary thyroid carcinoma²³, and oral squamous cell carcinoma²⁴. Moreover, it had been reported that

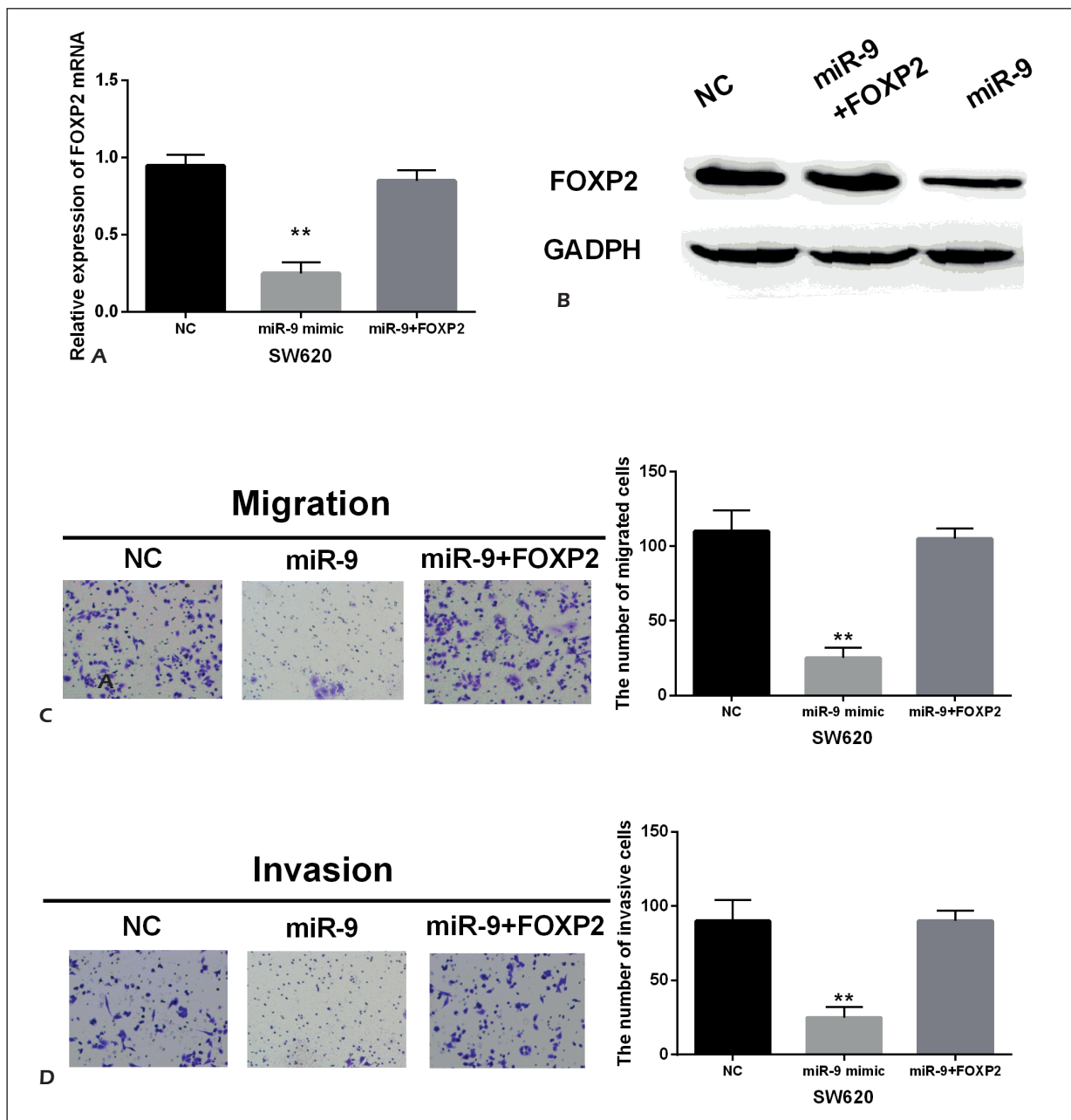


Figure 5. Overexpression of FOXP2 weakened the suppressive effect of miR-9-5p in CRC. **A-B**, The mRNA and protein expressions of FOXP2 were measured in SW620 cells with FOXP2 vector and miR-9-5p. **C-D**, Cell migration and invasion in SW620 cells with FOXP2 vector and miR-9-5p (magnification: 40 $\times$). ** $p < 0.01$.

miR-9 was downregulated in colorectal cancers. However, the specific role of miR-9 has not been investigated previously²⁵. Our research also proposed that miR-9-5p expression was decreased in CRC. Furthermore, we found that miR-9-5p exerted an inhibitory effect in the tumorigenesis of CRC.

More importantly, dysregulation of miR-9 had been detected to promote proliferation and tumor

cell survival in colon cancer²⁶. Zhu et al²⁷ reported that miR-9 could regulate cell proliferation and apoptosis in CRC. A follow-up study²⁸ indicated that up-regulation of miR-9 was involved in the metastasis of CRC. Moreover, miR-9 was identified to suppress cell migration and invasion by downregulating TM4SF1 in CRC²⁹. Similarly, our findings also suggested that miR-9-5p suppressed metastasis in CRC *via* inhibiting cell migration

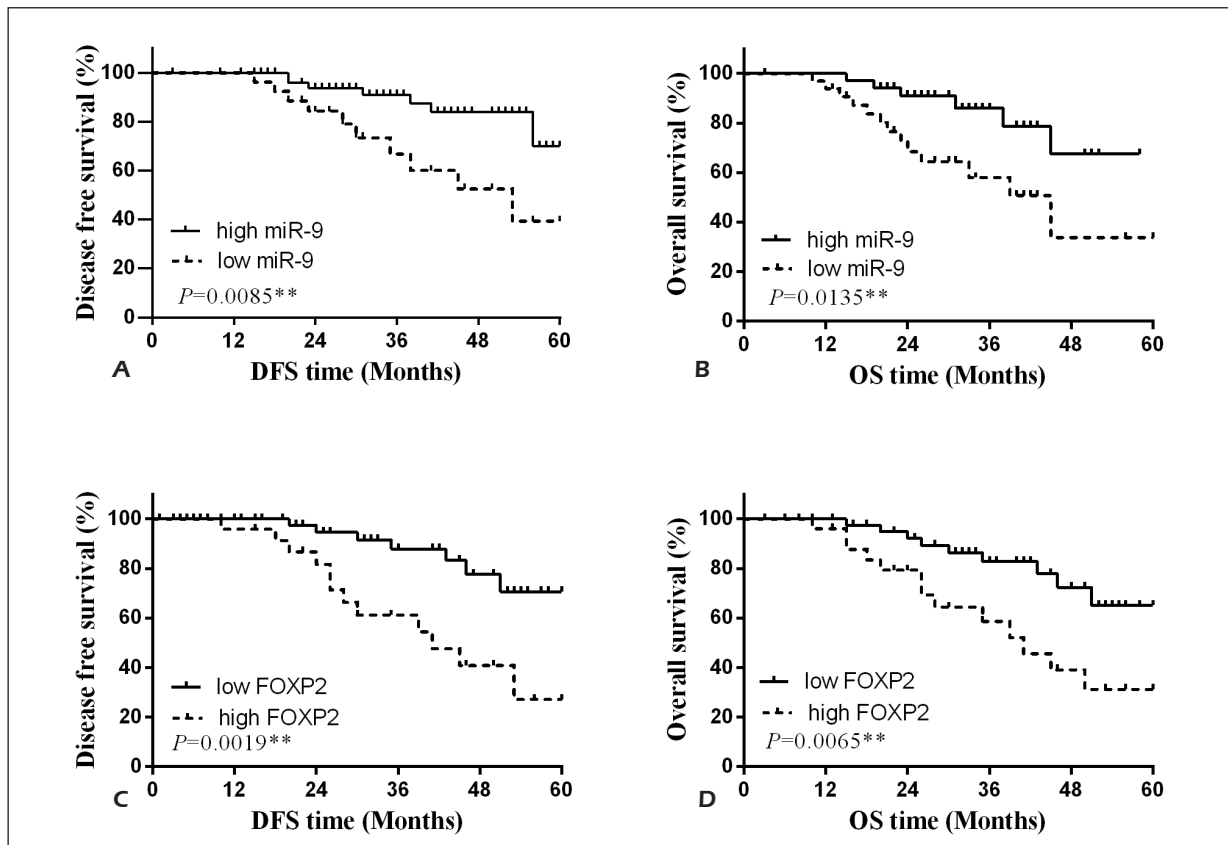


Figure 6. MiR-9-5p and FOXP2 could predict the prognosis of CRC patients. **A-B**, CRC patients with high miR-9-5p expression showed longer DSF and OS. **C-D**, CRC patients with low FOXP2 expression had longer DSF and OS. ** $p < 0.01$.

and invasion. We also found that miR-9-5p inhibited EMT. These results demonstrated that miR-9-5p acted as a tumor suppressor in tumorigenesis of CRC.

Additionally, the effect of miRNAs on EMT in CRC had been reported in previous studies. For instance, the upregulation of miR-186-5p was found to inhibit cell metastasis and EMT in CRC by targeting ZEB1³⁰. Similarly, Hu et al³¹ reported that miR-363-3p inhibited EMT and suppressed metastasis in CRC by targeting Sox4. It had been shown that miR-9 blocked EMT in colon cancer cells through promoting E-cadherin³². In this study, miR-9-5p was also detected to suppress EMT through promoting E-cadherin expression. Besides that, we observed that FOXP2 was a direct target gene of miR-9-5p.

Forkhead box P2 (FOXP2) that shares a common DNA-binding consensus sequence with other FOXP members belongs to the forkhead box P family³³. Many miRNAs have been reported to directly target FOXP2, such as miR-196b³⁴, miR-190³⁵, and miR-23a³⁶. In this study, we verified

the relation between FOXP2 and miR-9-5p. We demonstrated that miR-9-5p inhibited tumorigenesis of CRC by binding to FOXP2. In addition, FOXP2 was found to promote metastasis and EMT in CRC. Previous studies^{37,38} also demonstrated that FOXP2 promoted invasion and metastasis in human hepatocellular carcinoma and breast cancer cells, similar to our results. We also found that low expression of miR-9-5p and high expression of FOXP2 predicted poor prognosis in CRC patients, which has not been reported in previous studies.

Conclusions

We revealed that miR-9-5p suppressed cell metastasis and EMT in CRC through targeting FOXP2. Moreover, the dysregulation of miR-9-5p and FOXP2 can predict the prognosis of CRC patients. MiR-9-5p/FOXP2 axis will provide a new breakthrough in the diagnosis and treatment of CRC.

Competing Interests

The authors declare that they have no competing interests.

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