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MiRNA-802 inhibits the metastasis of colorectal cancer by targeting FOXE1

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of microRNA-802 (miRNA-802) in the progression of colorectal cancer (CRC) and the underlying mechanism.

PATIENTS AND METHODS: The relative expression levels of miRNA-802 and FOXE1 in 40 paired CRC tissues and adjacent normal tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between miRNA-802 expression and the pathological indexes of CRC patients was assessed. Meanwhile, the prognostic potentials of miRNA-802 and FOXE1 in CRC patients were identified through the Kaplan-Meier method. After overexpression of miRNA-802, the changes in the proliferative, migratory, and invasive capacities of HT29 and HCT-8 cells were evaluated *in vitro*. The Dual-Luciferase Reporter Gene Assay was applied to investigate the binding relationship between miRNA-802 and FOXE1. Finally, the rescue experiments were carried out to uncover the role of the miRNA-802/FOXE1 axis in regulating the cellular behaviors of CRC.

RESULTS: MiRNA-802 was significantly downregulated in CRC tissues and cell lines. CRC patients with a low level of miRNA-802 had significantly higher rates of lymphatic metastasis and distant metastasis, as well as worse overall survival. The transfection of miRNA-802 mimics remarkably attenuated the proliferation, migration, and invasion of HT29 and HCT-8 cells. FOXE1 expression was significantly upregulated in CRC tissues and cell lines. Meanwhile, the expression of FOXE1 was negatively correlated with miRNA-802 in CRC tissues. A higher level of FOXE1 indicated the worse prognosis of CRC patients. The Dual-Luciferase Reporter Gene Assay further verified the binding relationship between FOXE1 and miRNA-802. Importantly, the overexpression of FOXE1 could reverse the regulatory effects of miRNA-802 on the cellular behaviors of CRC.

CONCLUSIONS: MiRNA-802 is significantly downregulated in CRC, and is closely related to lymphatic and distant metastasis of CRC. Furthermore, miRNA-802 alleviates the malignant progression of CRC *via* negatively regulating FOXE1.

Key Words

MiRNA-802, FOXE1, Colorectal cancer (CRC), Metastasis.

Introduction

Colorectal cancer (CRC) is a common malignancy in the digestive system, seriously endangering human health. The incidence and development of CRC are closely related to heredity, lifestyle, and precancerous lesions[1,2]. It is estimated that CRC has become the third prevalent malignant tumor in the world. About two-thirds of CRC cases are from developed countries[3,4]. The incidence of CRC in the Chinese population is on the rise, whose onset age becomes much younger in recent years[5,6]. Previous studies have indicated that CRC usually affects people aged 40-70 years old. The symptoms of early-stage CRC are atypical. The vast majority of CRC patients have already been in an advanced stage or accompanied by metastasis when diagnosed[7,8]. Therefore, it is urgent to improve the diagnostic and therapeutic efficacies of CRC[9,10].

With significant advances in molecular biotechnology, a potential interaction between miRNAs and tumorigenesis has been well concerned. MicroRNAs (miRNAs) are promising targets for the clinical treatment of tumors[11,12]. MiRNAs are a type of non-coding, single-stranded RNAs encoded by endogenous genes. They are widely distributed in plants and animals. Meanwhile, miRNAs are functional molecules that can regulate the gene expression[13,14]. So far, 28,645 miRNAs have been discovered in human beings[15]. Every single miRNA can target several downstream genes, and a certain gene can be regulated by multiple miRNAs. This may eventually form a complex regulatory network[16,17]. By analyzing miRNA microarray of CRC, we found that miRNA-802 was significantly upregulated in invasive CRC cells. However, the specific biological function of miRNA-802 in CRC has rarely been reported[18].

Patients and Methods

CRC Samples

A total of 40 paired CRC tissues and adjacent normal tissues were collected from patients

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during radical resection. None of the enrolled CRC patients received preoperative anti-tumor therapies. The clinical indexes were collected from patients and were further analyzed. The informed consent was obtained from patients and their families. This study was approved by the Ethics Committee of Shanxi Cancer Hospital.

Cell Culture

CRC cell lines (HCT-116, HT29, HCT-8) and human colon cell line (FHC) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Except for HT29 cells which were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA), the other cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator. At 80-90% of confluence, the cell passage was conducted using 1×trypsin + ethylenediaminetetraacetic acid (EDTA).

Cell Transfection

The transfection plasmids were provided by GenePharma (Shanghai, China). The cells were pre-seeded into 6-well plates. The cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 70% of confluence. 48 h after transfection, the cells were harvested for verification of transfection efficacy and subsequent experiments.

Cell Counting Kit-8 (CCK-8) Assay

The cells were first seeded into 96-well plates at a density of 2×10^3 cells per well. At appointed time points, the absorbance value at 450 nm was recorded in accordance with CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan). Finally, the viability curve was plotted.

Transwell Assay

Firstly, the cells were adjusted to a concentration of 2.0×10^5/mL. 200 μL/well cell suspension was added to the upper side of the transwell chamber (Millipore, Billerica, MA, USA) coated with Matrigel. Meanwhile, 700 μL of medium containing 10% FBS was added to the lower chamber. After 48 h of incubation, the cells invaded to the bottom side were fixed with methanol for 15 min, and stained with crystal violet for 20 min. Then, the cells were observed under a microscope. The number of invasive cells was counted, and 5 fields were randomly selected for each sample (magnification 10×). The migration assay was conducted in the same way except for Matrigel pre-coating.

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. Subsequently, extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Obtained cDNAs were 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 for mRNA and miRNA, respectively. Each sample was performed in triplicate, and the relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: FOXE1, F: 5'-CCACGGAGACGCGCTGTACGTC-3', R: 5'-GCTGAGGGACTGTGTCCAGAAGCA-3'; microRNA-802, F: 5'-GCGTACAGACAGTCCGGACGTG-3', R: 5'-AGTTCGTGCTGGTGCGACCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

The total protein in the cells was extracted using RIPA (Beyotime, Shanghai, China). The concentration of the extracted protein was quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Later, the protein samples were separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skimmed milk for 2 hours, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with corresponding secondary antibodies for 2 h. The immunoreactive bands were exposed by the enhanced chemiluminescence (ECL) method and analyzed by Image Software (NIH, Bethesda, MD, USA).

Dual-Luciferase Reporter Gene Assay

The cells were first co-transfected with miRNA-802 mimics/NC and pmirGLO-FOXE1-WT/pmirGLO-FOXE1-MUT using Lipofectamine 2000. 24 hours later, the co-transfected cells were harvested. The Luciferase activity was finally determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. The experimental data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. The Kaplan-Meier curve was introduced for survival analysis. The Spearman regression test was performed to evaluate the relationship between the two genes. $p<0.05$ was considered statistically significant.

Results

MiRNA-802 Was Lowly Expressed in CRC Tissues and Cell Lines

Compared with adjacent normal tissues, miRNA-802 was significantly downregulated in CRC tissues (Figure 1A). Besides, miRNA-802 was lowly expressed in CRC cells when compared with FHC cells (Figure 1B). HT29 and HCT-8 cells expressed the lowest level of miRNA-802 among these CRC cell lines. Therefore, the two cell lines were chosen for establishing overexpression model of miRNA-802 *in vitro*.

MiRNA-802 Expression Was Correlated with Lymphatic Metastasis, Distant Metastasis and Overall Survival in CRC Patients

Based on the median level of miRNA-802, 40 enrolled CRC patients were divided into two groups, including high-level group and low-level group. The results showed that miRNA-802 expression was correlated with lymphatic metastasis and distant metastasis of CRC patients, whereas it was not associated with age, gender, and tumor staging (Table I). To uncover the relationship between miRNA-802 expression and the prognosis of CRC, the follow-up data were collected. The Kaplan-Meier curve revealed significantly worse survival in CRC patients with the low expression level of miRNA-802 (Figure 1C).

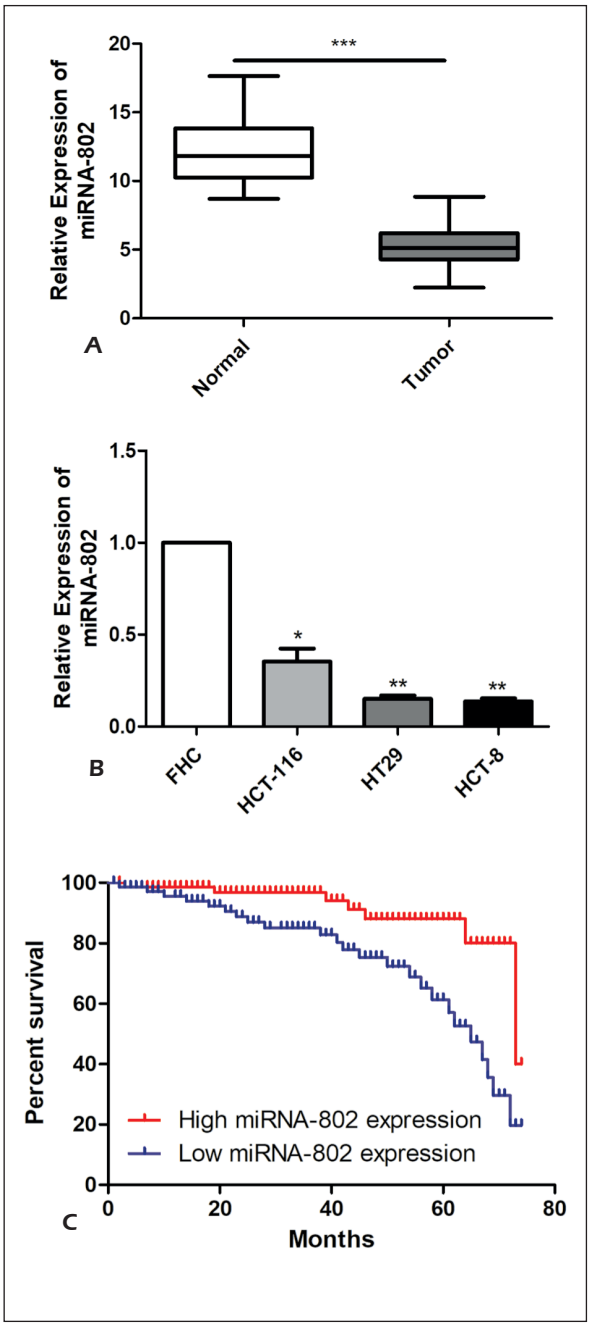

Figure 1. MiRNA-802 was lowly expressed in CRC tissues and cell lines. **A**, Relative level of miRNA-802 in CRC tissues and matched normal tissues (n=40). **B**, Relative level of miRNA-802 in FHC cells and CRC cells (HCT-116, HT29, HCT-8). **C**, Kaplan-Meier curves revealed the overall survival in CRC patients with high and low expression of miRNA-802.

Overexpression of MiRNA-802 Suppressed Proliferation, Migration, and Invasion of CRC Cells

The transfection of miRNA-802 mimics significantly upregulated miRNA-802 level in HT29 and HCT-8 cells (Figure 2A). The viability of HT29 and HCT-8 cells was markedly reduced after transfection of miRNA-802 mimics (Figure 2B). The transwell assay demonstrated the number of migratory and invasive HT29 and HCT-8 cells with miRNA-802 overexpression was significantly reduced (Figure 2C). Therefore, the overexpression of miRNA-802 could attenuate the proliferation, migration, and invasion of CRC.

Table I. Association of miRNA-802 expression with clinicopathologic characteristics of colorectal cancer.

Parameters	No. of cases	miRNA-802 expression		*p*-value*
		Low (%)	High (%)	
Age (years)				0.931
<60	13	8	5	
≥60	27	17	10	
Gender				0.567
Male	19	11	8	
Female	21	14	7	
T stage				0.673
T1-T2	25	15	10	
T3-T4	15	10	5	
Lymph node metastasis				0.015
No	27	21	6	
Yes	13	5	8	
Distance metastasis				0.006
No	30	21	9	
Yes	10	2	8	

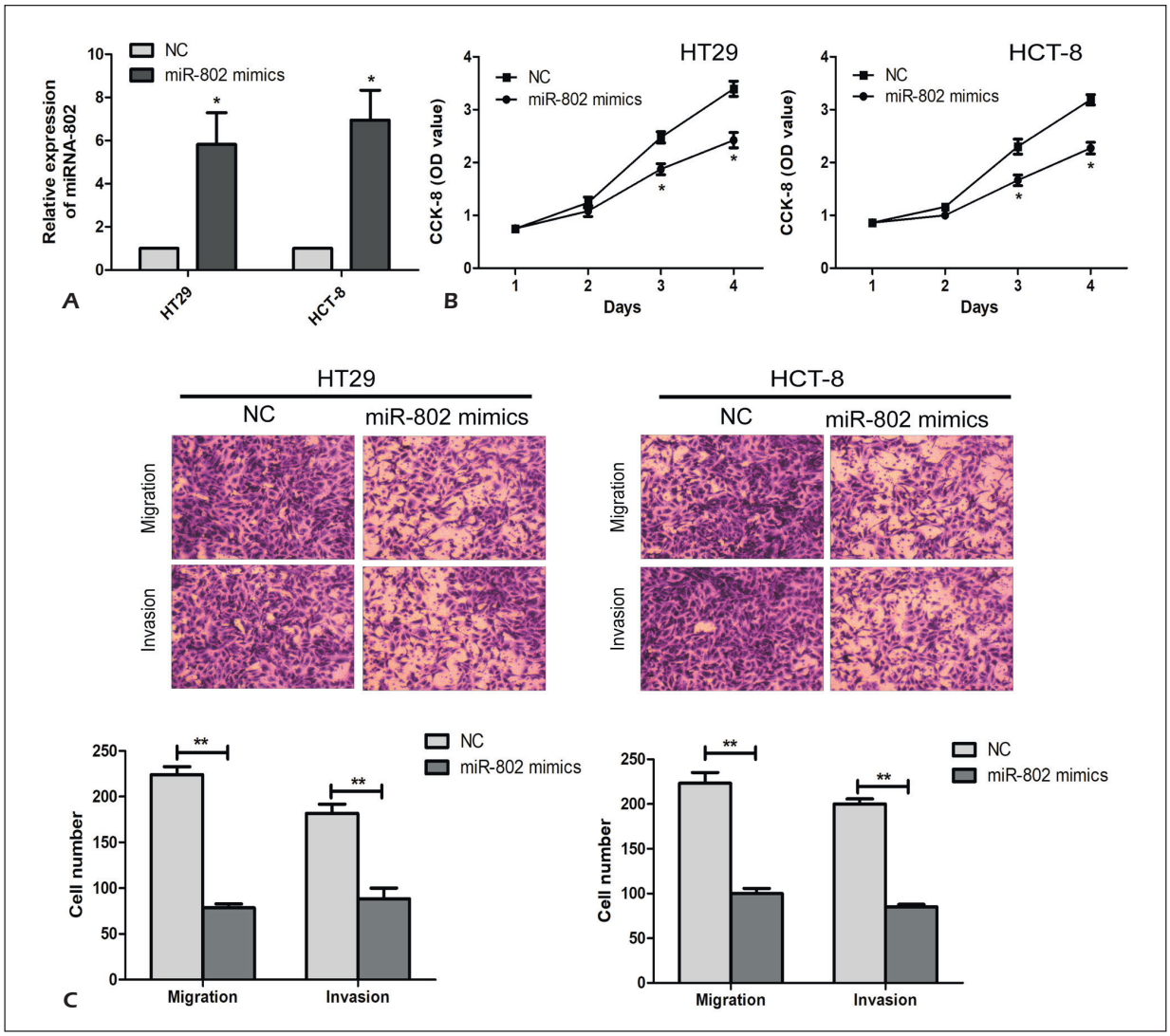

Figure 2. Overexpression of miRNA-802 suppressed the proliferation, migration, and invasion of CRC cells. **A**, Transfection efficacy of miRNA-802 in HT29 and HCT-8 cells. **B**, CCK-8 assay showed the viability of HT29 and HCT-8 cells transfected with NC or miRNA-802 mimics. **C**, The transwell assay showed the migratory and invasive abilities of HT29 and HCT-8 cells transfected with NC or miRNA-802 mimics (magnification 10×).

FOXE1 Was Highly Expressed in CRC Tissues and Cell Lines

The bioinformatics method predicted that FOXE1 was the target gene of miRNA-802. Its expression level in CRC tissues was significantly higher than adjacent normal tissues (Figure 3A). Similarly, FOXE1 was remarkably upregulated in CRC cells (Figure 3B). In 40 CRC tissues, the miRNA-802 level was negatively correlated with FOXE1 level (Figure 3C). Subsequently, the follow-up data of CRC patients were collected. The survival analysis depicted a remarkably worse prognosis of CRC patients with a high level of FOXE1 (Figure 3D).

MiRNA-802 Could Bind to FOXE1

Based on the binding sequences between FOXE1 and miRNA-802, pmirGLO-FOXE1-WT and pmirGLO-FOXE1-MUT were constructed for the Dual-Luciferase Reporter Gene Assay. The co-transfection of pmirGLO-FOXE1-WT and miRNA-802 mimics resulted in significantly decreased Luciferase activity in HT29 and HCT-8 cells. This confirmed the binding relationship between miRNA-802 and FOXE1 (Figure 4A). The transfection of miRNA-802 mimics in CRC cells significantly downregulated both the mRNA and protein levels of FOXE1 (Figures 4B, 4C).

MiRNA-802 Modulated FOXE1 Expression in CRC Cells

To clarify the involvement of FOXE1 in the miRNA-802-influenced progression of CRC, rescue experiments were conducted. The overexpression of FOXE1 markedly upregulated FOXE1 level in CRC cells (Figure 5A). The viability, migration, and invasion of CRC cells were markedly promoted after the overexpression of FOXE1. Notably, attenuated proliferative, migratory, and invasive capacities of CRC cells overexpressing miRNA-802 could be partially reversed by FOXE1 overexpression (Figures 5B, 5C).

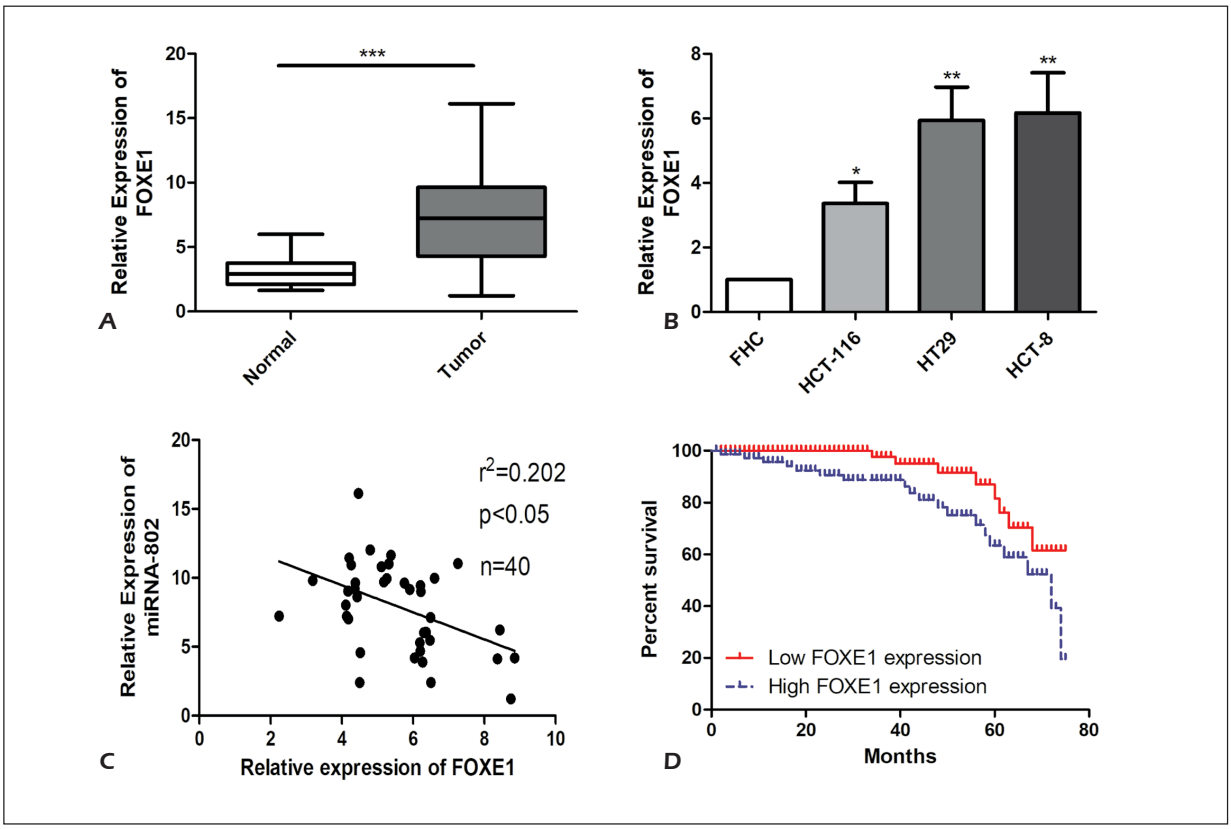

Figure 3. FOXE1 was highly expressed in CRC tissues and cell lines. **A**, The relative level of FOXE1 in CRC tissues and matched normal tissues (n=40). **B**, Relative level of FOXE1 in FHC cells and CRC cells (HCT-116, HT29, HCT-8). **C**, A negative correlation between expression levels of miRNA-802 and FOXE1 in CRC tissues. **D**, Kaplan-Meier curves revealed the overall survival in CRC patients with high and low expression of FOXE1.

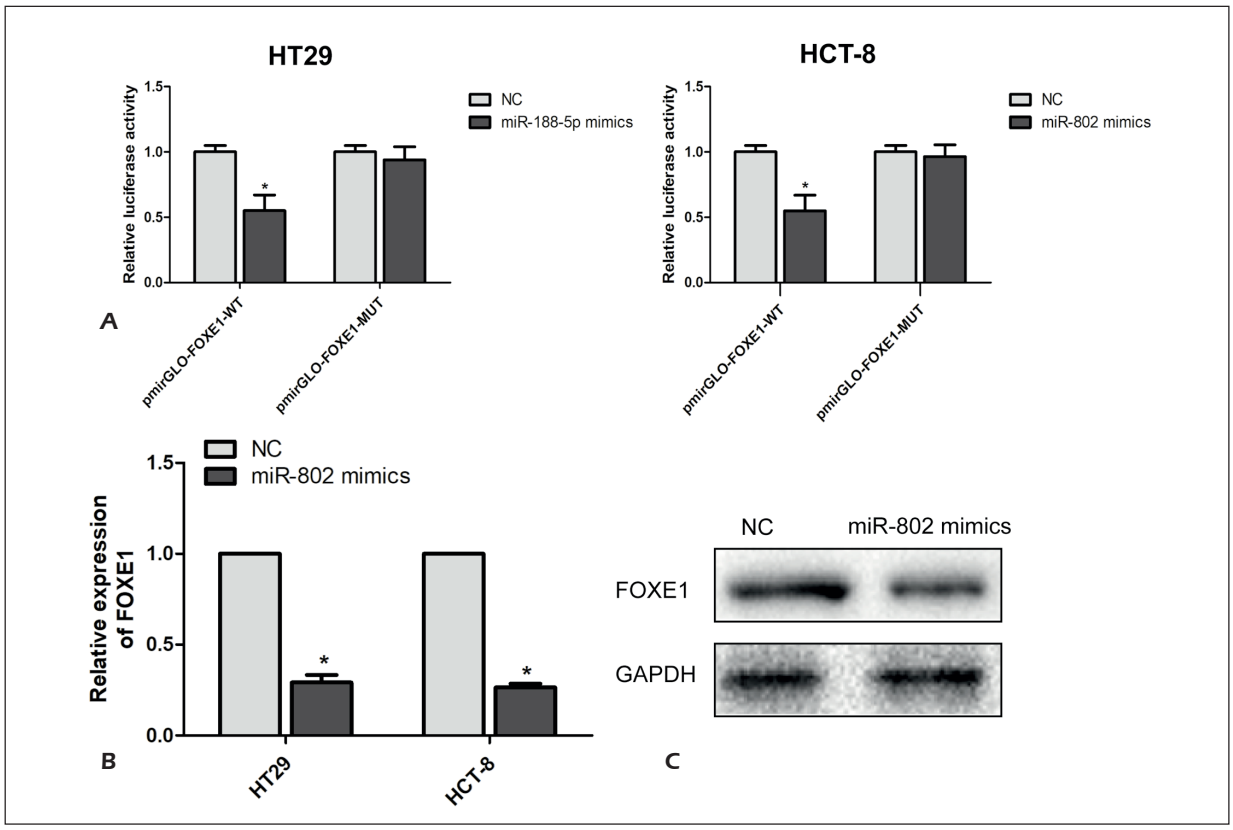

Figure 4. MiRNA-802 could bind to FOXE1. **A**, Dual-Luciferase Reporter Gene Assay showed relative Luciferase activity in HT29 and HCT-8 cells co-transfected with miRNA-802 mimics/NC and pmirGLO-FOXE1-WT/pmirGLO-FOXE1-MUT. **B**, The relative level of FOXE1 in HT29 and HCT-8 cells transfected with NC or miRNA-802 mimics. **C**, The protein level of FOXE1 in HT29 and HCT-8 cells transfected with NC or miRNA-802 mimics.

Discussion

Currently, except for a small number of CRC patients with evident familial morbidity, most of them are sporadic. The pathogenesis of CRC is related to individual gene mutations, including chromosomal mutations and DNA mismatch repair[4-6]. In addition to the primary lesion, the infiltration and metastasis foci of CRC seriously endanger human health[7]. Local invasion is the most common infiltration form of CRC. The tumor gradually infiltrates surrounding tissues or organs through implantation and metastasis, resulting in the corresponding clinical symptoms[7,8]. So far, multiple signaling pathways have been found closely related to the development of CRC, including Wnt/β-catenin, Ras, and p53 pathways[9,10].

MiRNAs are a type of non-coding RNAs with 18-25 nucleotides in length[11,12]. In human genes, miRNAs account for only 1%. However, they are capable of regulating the expression, modification, translation, and transcription of over 1/3 genes[12-14]. Previous studies have shown that miRNAs are widely involved in the development of tumors by targeting oncogenes or tumor-suppressor genes. One dysregulated miRNA can result in a series of cascade reactions and feedback pathways involving multiple mRNAs and target genes. Meanwhile, multiple miRNAs can target the same gene, thereby regulating the expression of this gene[15-17]. In this study, miRNA-802 was found significantly downregulated in CRC tissues. Its expression level was negatively correlated with the metastatic rate of CRC. Hence, we believed that miRNA-802 exerted a carcinogenic role in the progression of CRC. *In vitro* abundance of miRNA-802 was identically lower in CRC cell lines. The overexpression of miRNA-802 mimics remarkably attenuated the proliferation, migration, and invasion of HT29 and HCT-8 cells.

The Forkhead Box Family is a large family of transcription factors, which is characterized by a distinct Forkhead region in the molecular structure[19,20]. Various members of this family are the

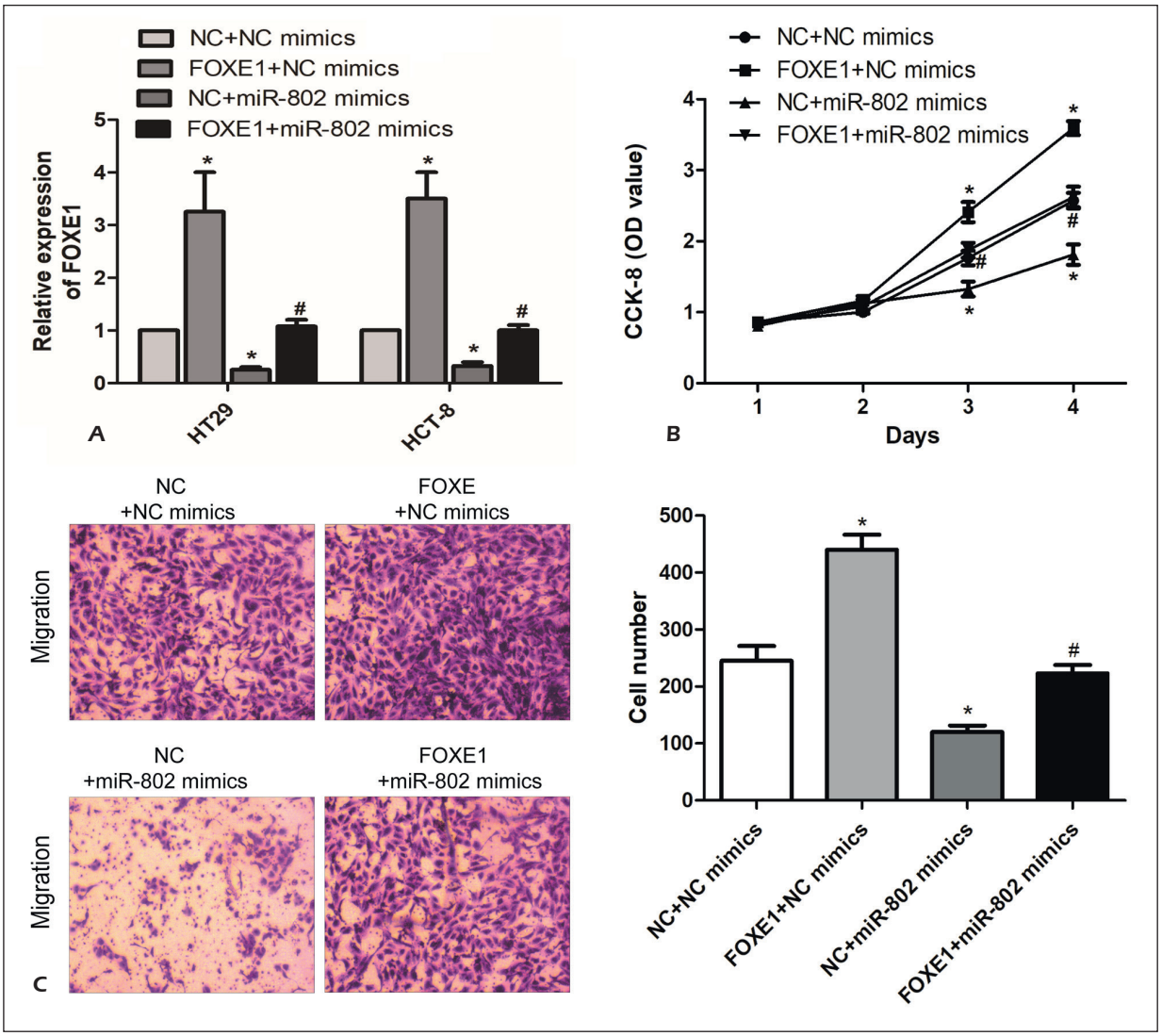

Figure 5. MiRNA-802 modulated FOXE1 expression in CRC cells. HT29 and HCT-8 cells were transfected with NC + NC mimics, pcDNA-FOXE1 + NC mimics, NC + miRNA-802 mimics, and pcDNA-FOXE1 + miRNA-802 mimics, respectively. **A**, The relative level of miRNA-802. **B**, CCK-8 assay showed the viability of cells. **C**, The transwell assay showed the migratory and invasive abilities of the cells (magnification 10×).

key regulators of cellular functions and tumor biology[19,20]. Forkhead box protein E1 (FOXE1) is one of the most specific transcription factors. FOXE1 is widely recognized as an activator in adult growth and development. In addition, FOXE1 is involved in cell growth and differentiation[20,21]. Many reports[22,23] have confirmed the decisive role of FOXE1 in the initial stages of certain tumors. The bioinformatics and subsequent Dual-Luciferase Reporter Gene Assay predicted and verified that FOXE1 was the target downstream of miRNA-802 in CRC. FOXE1 was remarkably upregulated in CRC tissues and cell lines. Meanwhile, its level was negatively correlated with miRNA-802 in CRC tissues. A higher level of FOXE1 indicated the significantly worse prognosis of CRC patients. Of note, the overexpression of FOXE1 reversed the regulatory effects of miRNA-802 on cellular behaviors of CRC.

Conclusions

We first identified that miRNA-802 is significantly downregulated in CRC, and is closely correlated with lymphatic and distant metastasis of CRC. Furthermore, miRNA-802 alleviates the malignant progression of CRC *via* negatively regulating FOXE1.

Conflict of Interests

The authors declare that they have no conflict of interests.

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