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MiRNA-212 acts as a tumor-suppressor in colorectal carcinoma through targeting SOX4

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Abstract. – OBJECTIVE: Colorectal cancer is a common gastrointestinal cancer, with mortality ranking the third all over the world. MicroRNA-212 (miR-212), located on chromosome 17p13.3, was lowly expressed in a variety of tumors. The purpose of our study was to explore the effects of miR-212 on colorectal cancer (CRC) cells.

PATIENTS AND METHODS: Quantitative Real Time-Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and Western blot were employed to evaluate the levels of mRNAs and proteins. The transwell assay was performed to calculate the abilities of migration and invasion. The Luciferase reporter assay was utilized to verify miR-212 targeting to the 3'-UTR of SOX4 mRNA. Statistical analysis was applied to analyze the experimental data.

RESULTS: MiR-212 was lowly expressed in CRC tissues and cell lines, while the expression of SRY-box 4 (SOX4) was overexpressed. Exogenous increasing of miR-212 or knockdown of SOX4 inhibited the migration, invasion and epithelial-mesenchymal transition (EMT) in CRC cells. Moreover, the expression of miR-212 had a negative connection with SOX4 expression, and miR-212 mediated the expression of SOX4 by directly binding to the 3'-UTR of SOX4 mRNA. In addition, low expression of miR-212 or overexpression of SOX4 was associated with poor prognosis of colorectal cancer patients.

CONCLUSIONS: MiR-212 inhibited the migration, invasion and EMT by direct targeting to the 3'-UTR of SOX4 mRNA in colorectal cancer. The newly identified miR-212/SOX4 axis provides novel insight for colorectal treatment.

Key Words:
MiR-212, SOX4, EMT, Colorectal cancer.

Introduction

Colorectal cancer (CRC), a gastrointestinal cancer, is the third most common cancer. About 600,000 cases die of CRC annually[1,2]. Although it has established surgical techniques and adjuvant therapies, the clinical outcome of CRC patients remains poor because of the high local tumor recurrence and distant metastases[3-6]. Thus, it is urgent to verify useful biomarkers for early diagnosis and the treatment of CRC.

MicroRNAs (miRNAs), a family of short non-coding RNAs, could inhibit the expression of target genes by binding to the 3'-untranslated region (3'-UTR) of mRNA[7,8]. Scholars[9-12] demonstrated that miRNAs played important roles in colorectal cancer, including miR-185, miR-335, miR-129 and miR-221. MiR-212, located on chromosome 17p13.3, has been reported to play great functions in several tumors included colorectal cancer[13]. In the prostate, Hu et al[14] discovered that miR-212 suppressed the proliferation and invasion by targeting to MAPK1. Similar findings by Gu et al[15] discovered that miR-212 inhibited the proliferation, migration and invasion of renal cell carcinoma by targeting to XIAP. Moreover, miR-212 acted as a tumor suppressor that could inhibit epithelial-mesenchymal transition (EMT) in thyroid cancer and inhibit triple negative breast cancer[16,17]. Therefore, we strongly believe that miR-212 could suppress the migration, invasion and EMT in colorectal cancer.

SRY-box 4 (SOX4) belongs to sex-determining region Y (SOX) family that could act as a transcriptional regulator after forming a protein complex together with other proteins. SOX4 is overexpressed in kinds of tumors including CRC, and it usually has a correlation with cell apoptosis and tumor angiogenesis[18,19]. In melanoma, Cheng et al[20] discovered that SOX4 could promote the migration and invasion by activating the NF-κB signaling pathway. According to Dai et al[21],

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SOX4 promoted proliferation signals through glycolysis by regulating AKT activation in melanoma cells. Furthermore, SOX4 has been reported to be overexpressed in renal cell carcinoma, that promoted cell migration and invasion inducing EMT[22]. In addition, SOX4 has been discovered to participate in metastasis and EMT in renal cell carcinoma, lung adenocarcinoma and non-small cell lung cancer[23-25]. Thus, we proposed that knockdown of SOX4 could inhibit migration, invasion and induced EMT of CRC cells.

Patients and Methods

Patients and Clinical Samples

Pairs of colorectal cancer and corresponding paracancerous clinical specimens were obtained from 53 CRC patients who received surgery at the Nanfang Hospital, Southern Medical University from January 2015 to December 2017. All the patients had not received any treatment before a surgical operation, and the excised tissues were immediately frozen in liquid nitrogen and stored at -80°C. We obtained informed consent from every patient who was participating in the present study. The approval for the experiments involving patient samples was obtained from the Ethics Committee of the Nanfang Hospital, Southern Medical University.

Cell Lines and Cell Culture

The human colorectal cell lines (LOVO and SW480) and normal colorectal epithelial cell line CC-18Co were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was employed to maintain all the cells, which were cultured at 37°C with a humidified atmosphere of 5% CO_2.

RNA Isolation and Quantitative Real Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the MIRcute and Separation of miRNAs Kit (Tiangen, Beijing, China) were applied to extract total RNAs and total miRNAs from tissues and cells. Reverse transcription reactions were performed by the Transcriptional First Strand complementary deoxyribose nucleic acid (cDNA) Synthesis kit (Roche, Basel, Switzerland), while the SYBR-Green Polymerase Chain Reaction Master Mix (Applied Biosystems, Foster City, CA, USA) was employed to perform Real Time-Polymerase Chain Reaction (RT-PCR). The internal reference of miR-212 and SOX4 were U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The specific primers are as follows: miR-212 Forward: 5'-CCTCGACTGGGGGTGTAAACAT-3', Reverse: 5'-GTGGAGTCGATTGCGTGTC-3'; U6 Forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', Reverse: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH Forward: 5'-TCAACGACCACTTTGTCAAGCTCA-3'; Reverse: 5'-GCTGGTGGTCCAGGGGTCTTACT-3'; SOX4 Forward: 5'-AGCGACAAGATCCCTTTCATTC-3'; Reverse: 5'-CGTTGCCGGACTTCACCTT-3'. The levels of miRNA and mRNA were calculated based on the $2^{-\Delta\Delta Ct}$ method.

Protein Extraction and Western Blotting

Radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) containing phosphatase inhibitor was employed to extract the total protein from tissues and cell lines. BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to quantify the protein concentration. The total proteins were separated by 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) through electrophoresis followed by electrotransfer on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were incubated in primary antibodies that included SOX4 (1:1000), E-Cadherin (1:1000), N-Cadherin (1:1000) and Vimentin (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight. The membranes were washed three times in Tris-Buffered Saline with Tween-20 (TBST; Sigma-Aldrich, St. Louis, MO, USA), and the membranes were incubated with secondary antibody with horseradish peroxidase (HRP)-conjugated (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. The enhanced chemiluminescence Kit (Amersham, Little Chalfont, UK) was applied to visualize the protein bands.

Cell Transfection

Vectors, including the miR-212 mimic, the miR-212 inhibitor, pcDNA3.1-SOX4 and siRNA-SOX4, as well as their negative control, were purchased from GenePharma (Shanghai, China). The miR-212 mimic and inhibitor were utilized to gain or loss of the expression of miR-212, whereas pcDNA3.1-SOX4 and siRNA-SOX4 were performed

to overexpress or knockdown SOX4. The transfection was performed by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 6-well plates.

Transwell Assays

Transwell assays without or with Matrigel were utilized to investigate the abilities of migration and invasion in colorectal cancer cells. Before the experiment, transwell chambers were placed in a 24-well plate and formed the upper and lower chamber. The upper chamber was seeded with 200 μL of cells suspension suspended in basal RPMI-1640, while the lower chamber was filled with 600 μL of RPMI-1640 supplemented with 20% FBS, that acted as an induction factor. After culturing for 48 h, we removed the cells still stayed on the upper surface using a cotton swab, fixed and stained the cells with methanol and crystal violet. We counted the cell number that moved to the underside of the membranes under a microscope.

Plasmid Construction and Luciferase Reporter Assay

TargetScan (http://www.targetscan.org/vert_71/) was conducted to predict the potential target gene of miR-212, and SOX4 was a candidate. To verify whether miR-212 bind to SOX4, we mutated the complementary sequences of miR-212 on the mRNA of SOX4 from GACUGUU to CUGACAA. Then, the wild-type sequence and the mutant were inserted into the pmirGlo vector, designated as SOX4-3'-UTR-WT (WT) and SOX4-3'-UTR-MUT (MUT), respectively. The miR-212 mimic or the NC mimic and WT or MUT were co-transfected in colorectal cells, and then the Luciferase activity was compared. The Luciferase activities of Firefly were calculated by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and the Renilla Luciferase activity was used as an internal reference.

Statistical Analysis

All the data were presented as the mean ± standard deviation and analyzed by Statistical Product and Service Solutions (SPSS) software 16.0 (Chicago, IL, USA) and GraphPad Prism 6.0 (La Jolla, CA, USA). The differences between two or more groups were analyzed by two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference). Pearson's correlation analysis was utilized to state the connection between the expression of miR-212 and SOX4. The Chi-square test was applied to analyze the connection between miR-212 and the clinicopathological data. The overall survival was evaluated by the Kaplan-Meier method and the log-rank test. $p<0.05$ was considered to be statistically significant. All experiments were performed in triplicate.

Results

The Expression of MiR-212 in Colorectal Cancer Tissues and Cell Lines

The mRNA level of miR-212 was evaluated in 53 pairs of CRC and the corresponding paracancerous

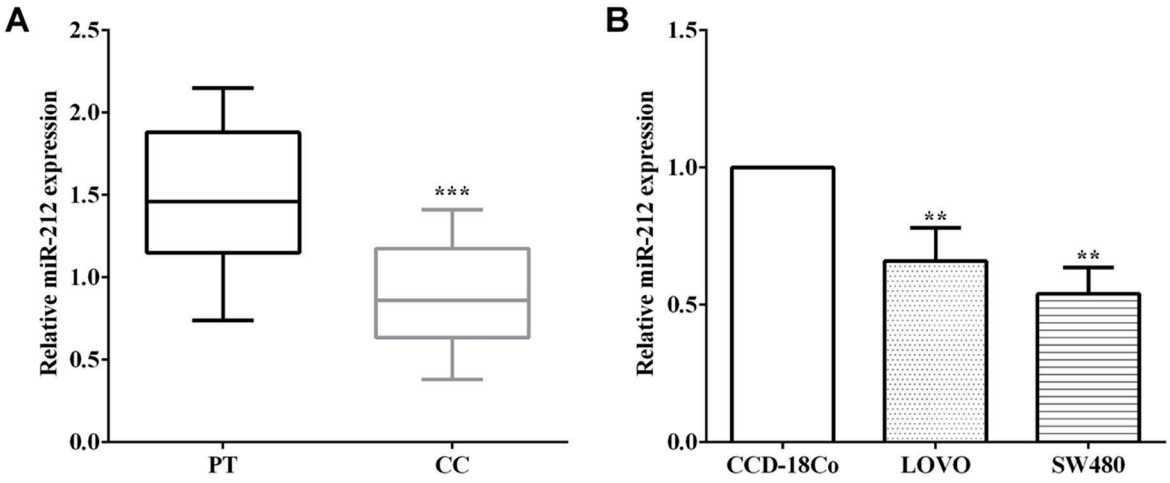

Figure 1. The expression of miR-212 in colorectal cancer tissues and cell lines. **A,** MiR-212 was upregulated in CRC tissues compared with the corresponding paracancerous tissues. **B,** MiR-212 was lowly expressed in CRC cell lines LOVO and SW480 compared with that measured in normal colorectal epithelial cell line CCD-18Co.

tissues by RT-qPCR. We discovered that miR-212 was markedly downregulated in all CRC samples compared with the corresponding paracancerous tissues ($p<0.0001$; Figure 1A). The expression of miR-212 was further examined in CRC cell lines CRC cells LOVO, also in normal colorectal epithelial cell line CCD-18Co. Similarly, the expression of miR-212 was lower in CRC cell lines LOVO ($p=0.0080$) and SW480 ($p=0.0011$) than that in normal colorectal epithelial cell CCD-18Co (Figure 1B).

MiR-212 Suppressed the EMT-Mediated Migration and Invasion of CRC Cells

To investigate the potential role of miR-212, the miR-212 mimic and the miR-212 inhibitor were used to gain- ($p=0.0020$) and loss-of ($p=0.0089$) the expression of miR-212 in CRC cell line SW480 (Figure 2A). The migration ($p=0.0008$) and invasion ($p=0.0004$) capacities were calculated by the transwell assay, and we found that they were significantly reduced by transfecting with the miR-212 mimic in SW480 cells. In contrast, the situation would be rescued by the miR-212 inhibitor, and the miR-212 inhibitor remarkably improved the cell migration ($p=0.0102$) and invasion ($p=0.0713$) abilities in SW480 cells (Figure 2B).

The EMT markers E-cadherin, N-cadherin and Vimentin were assessed by Western blot, to further demonstrate the potential role of miR-212 in modulating CRC metastasis. We discovered that miR-212 mimic could improve the expression of epithelial marker E-cadherin while inhibited the mesenchymal marker N-cadherin and Vimentin expression. To the contrary, with knockdown of miR-212, the expression of E-cadherin was decreased, whereas the expression of N-cadherin and Vimentin were increased (Figure 2C), which suggests that miR-212 suppressed the EMT of CRC cells.

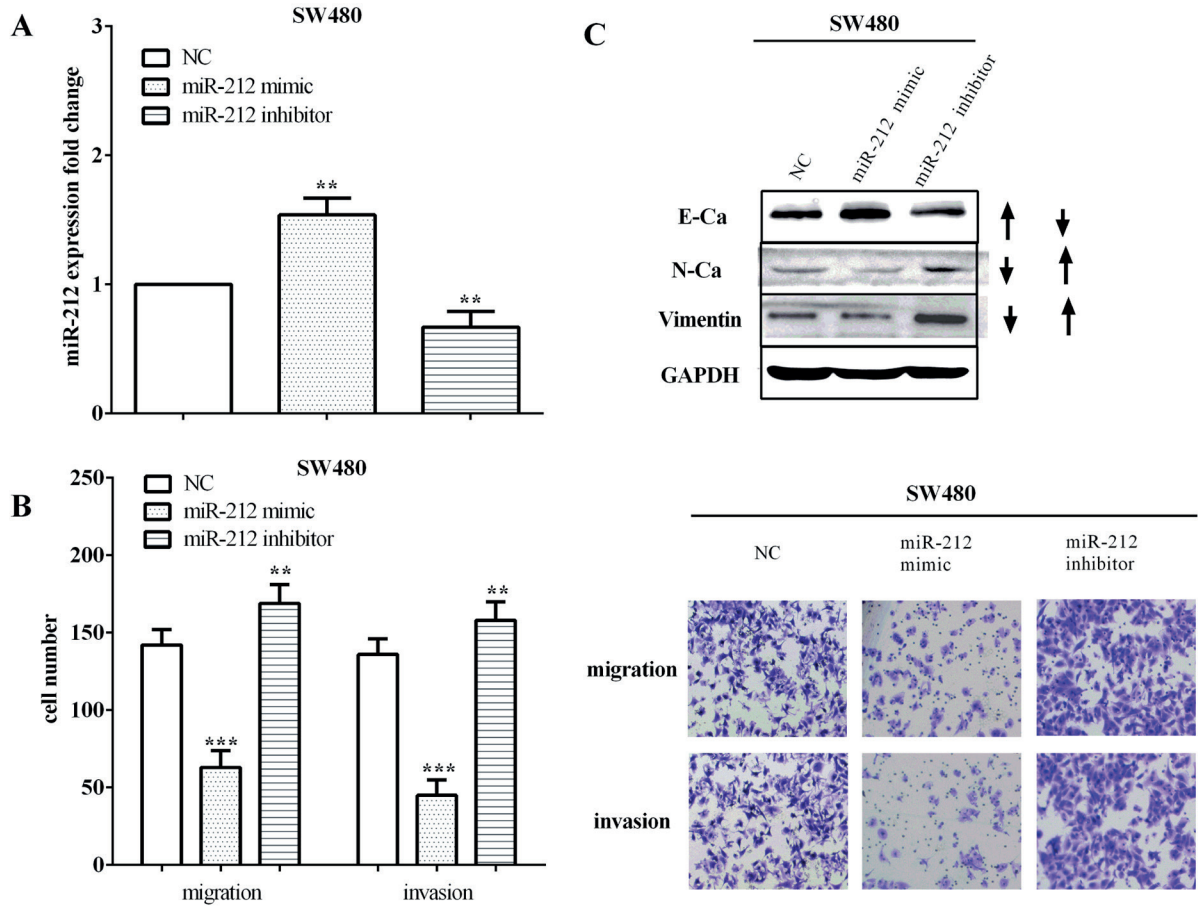

Figure 2. MiR-212 suppressed the migration and invasion-mediated EMT of CRC cells. **A,** The miR-212 mimic and inhibitor were performed to overexpression or knockdown miR-212 experiments in CRC cell line SW480. **B,** The migration and invasion capacities were reduced by transfecting with the miR-212 mimic, while improved by the miR-212 inhibitor in SW480 cells (Magnification × 40). **C,** Overexpression of miR-212 suppressed EMT, whereas knockdown miR-212 promoted the EMT in SW480 cells.

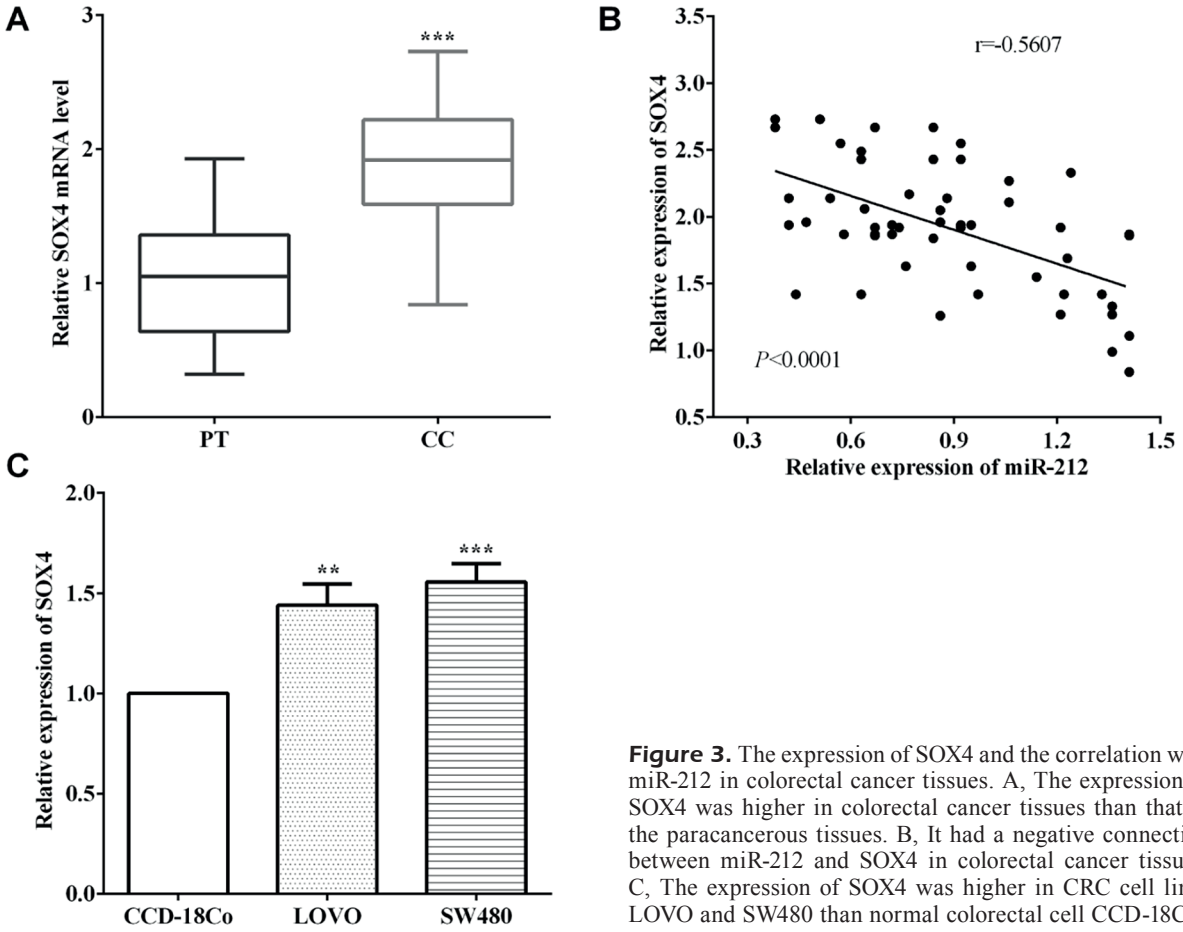

Figure 3. The expression of SOX4 and the correlation with miR-212 in colorectal cancer tissues. A, The expression of SOX4 was higher in colorectal cancer tissues than that in the paracancerous tissues. B, It had a negative connection between miR-212 and SOX4 in colorectal cancer tissues. C, The expression of SOX4 was higher in CRC cell lines LOVO and SW480 than normal colorectal cell CCD-18Co.

The Expression of SOX4 and the Correlation With MiR-212 in Colorectal Cancer Tissues

The expression of SOX4 in 53 pairs of colorectal cancer and the corresponding paracancerous tissues were evaluated by RT-qPCR, and we discovered that the expression of SOX4 was remarkably higher (p<0.0001) in colorectal cancer tissues than that in the corresponding paracancerous tissues (Figure 3A). Therefore, the correlation of the expression of miR-212 and SOX4 in colorectal cancer tissues. We found it had a negative connection between the expression of miR-212 and SOX4 (p<0.0001, r=-0.5607; Figure 3B). Moreover, the mRNA level of SOX4 in cell lines was calculated either, and we demonstrated that the expression of SOX4 was higher in CRC cell lines LOVO (p=0.0019) and SW480 (p=0.0004) than normal colorectal cell line CCD-18Co (Figure 3C).

Knockdown of SOX4 Inhibited the Migration and Invasion-Mediated EMT

To explore the functions of SOX4 on CRC cell progress, SW480 cells were transfected with siRNA-SOX4 to interfere with the expression of SOX4, and siRNA-NC acted as the negative control. The transfection efficiency was evaluated by RT-qPCR (p=0.0083; Figure 4A) and Western blot (Figure 4B). Then, we assessed the capacities of migration and invasion by transwell assay; as expected, interfering SOX4 reduced the number of migration (p=0.0065) and invasion (p=0.0060) in SW480 cells (Figure 4C). In addition, the expression of EMT markers was measured, and we found that the epithelial marker E-cadherin was remarkably improved while the mesenchymal markers N-cadherin and Vimentin were significantly decreased in SW480 cells, suggesting that ablation of SOX4 could inhibit colorectal cancer cell EMT (Figure 4D). All the results showed that

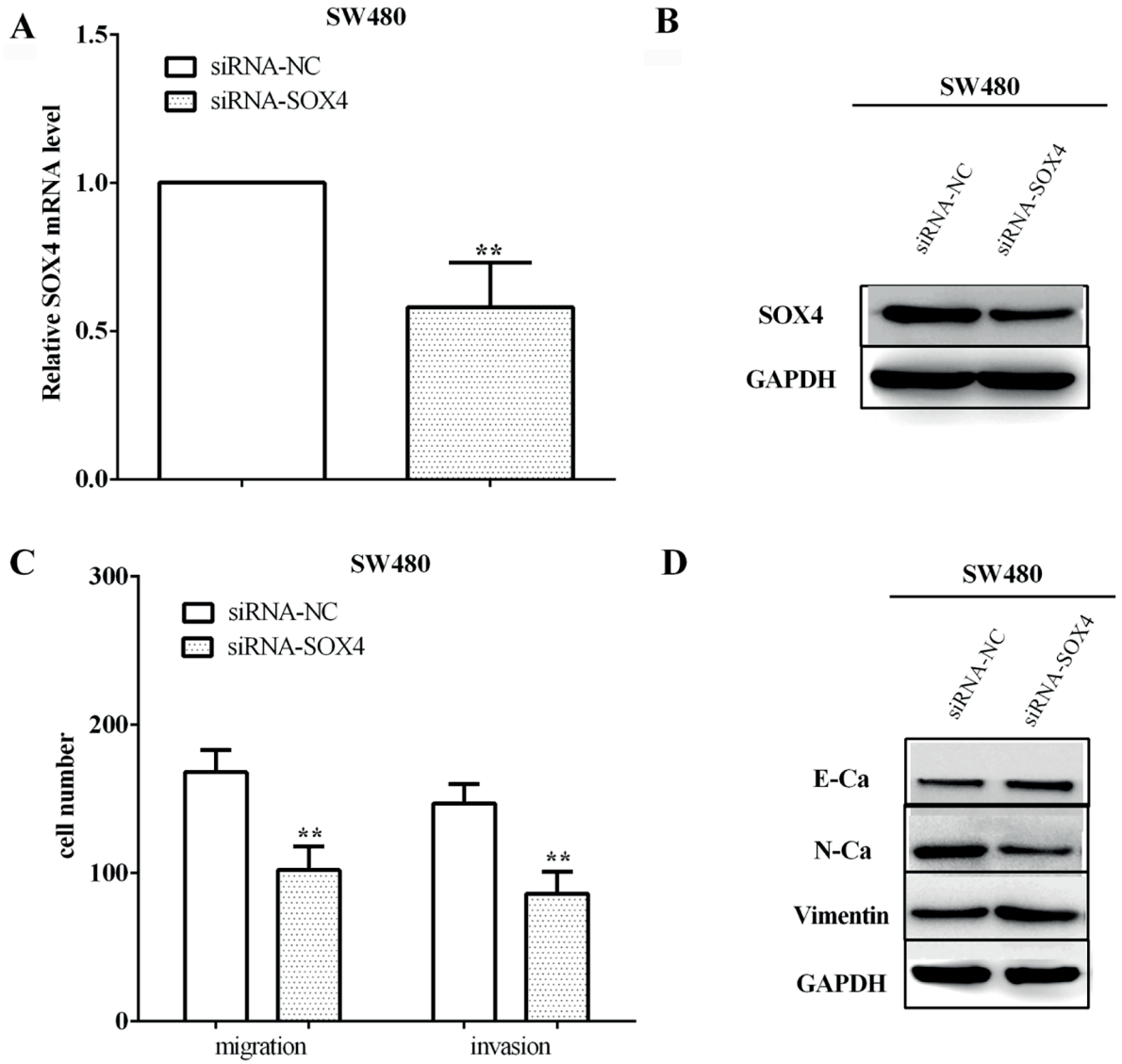

Figure 4. Knockdown of SOX4 inhibited the migration and invasion-mediated EMT. RT-PCR. **A,** and Western blot B, evaluated the interference effects of transfecting siRNA-SOX4 and siRNA-NC in SW480 cells. **C,** The number of migration and invasion were reduced after interfering SOX4 in SW480 cells. **D,** SOX4 ablation could inhibit colorectal cancer cell EMT.

knockdown of SOX4 could eliminate the effects of miR-212 overexpression on the migration, invasion and EMT.

MiR-212 Targeted to SOX4 and Regulated the Expression of SOX4 in CRC Cells

Due to the same effects of SOX4 downregulation and miR-212 upregulation on CRC cell progress, we wonder whether there is a direct link between them. TargetScan predicted that SOX4 was a direct target gene of miR-212 and provided the complementary sequences between miR-212 and SOX9. To verify whether miR-212 directly bind to the 3'-UTR of SOX4 mRNA in CRC cell lines, the binding sequences on SOX4 were mutated from GACUGUU (WT) to GACCCCU (MUT) (Figure 5A). Then, we co-transfected the miR-212 mimic and WT or MUT, and a Dual-Luciferase reporter assay was used to calculate the Luciferase activity. As expected, miR-212 mimic could significantly reduce (p=0.0042) the Luciferase activity of the wild-type 3'-UTR of SOX4 mRNA, whereas it failed to inhibit the Luciferase activity of cells

that transfected with the mutated 3'-UTR of SOX4 mRNA (p=0.4106; Figure 5B).

Moreover, the upregulation of miR-212 could inhibit (p=0.0017) the mRNA level of SOX4, while the downregulation of miR-212 increased (p=0.0021) the expression of SOX4 in SW480 cells (Figure 5C). These results demonstrated that miR-212 inhibits the expression of SOX4 by directly binding to its mRNA 3'-UTR.

Identification of MiR-212 and SOX4 Associated With Poor Survival in Colorectal Cancer

To investigate the clinical significance of miR-212 in CRC, the patients were divided into two groups according to the median of miR-212 expression. As shown in Table I, downregulation of miR-212 was negatively correlated with TNM stage (p=0.037), lymph node metastasis (p=0.017) and the expression of SOX4 (p=0.020), while it had a tendency to have a correlation with the tumor size (p=0.072) and metastasis (p=0.077). In addition, the Kaplan-Meier analysis demonstrated that the patients with low expression of miR-212 had a shorter 5-year overall survival (OS) than the miR-212 high expression patients (p=0.0244; Figure 6A). On the contrary, overexpression of SOX4 was associated with poor prognosis (p=0.0302; Figure 6B), which indicated that downregulation of miR-212 or upregulation of SOX4 could predict poor outcome of colorectal cancer patients.

Discussion

Colorectal cancer (CRC) is the third most common malignancy, with about 600,000 cases who die of CRC annually[1,2]. The metastasis is the major cause of serious morbidity and mortality in CRC patients and because of the high local tumor recurrence and distant metastases, the clinical outcome of CRC patients remains poor[3-6,26]. Thus, it is urgent to verify useful biomarkers for early diagnosis and metastasis of CRC. Increasing evidence demonstrated that miRNAs may function as biomarkers and they played important roles in colorectal cancers[9-12]. MiR-212 has been reported to

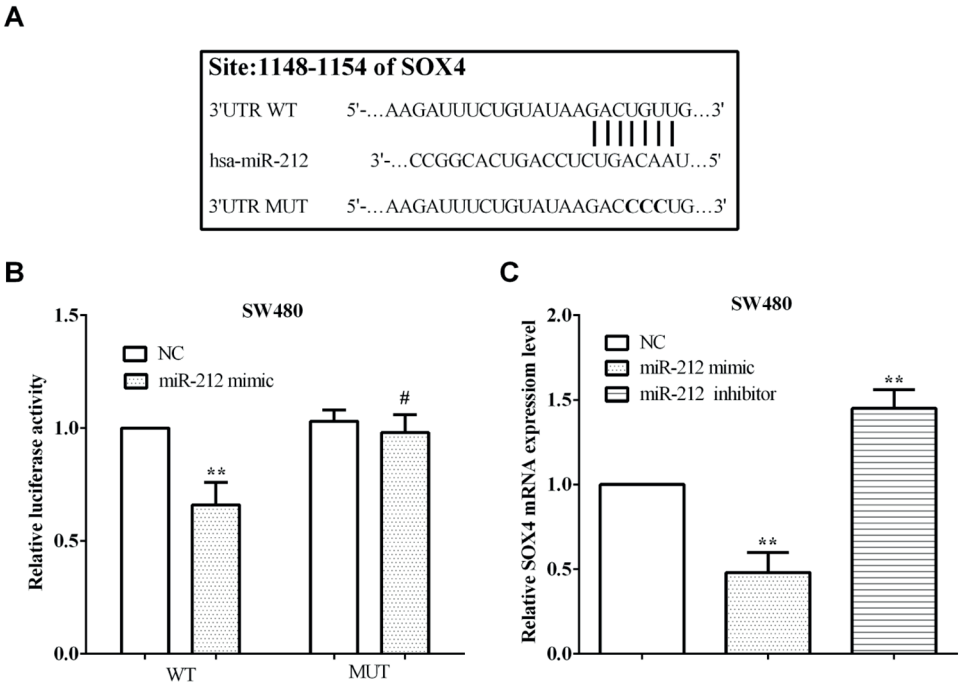

Figure 5. MiR-212 directly targeted to the 3'-UTR of SOX4 mRNA and regulated the expression of SOX4 in CRC cell lines. **A,** The complementary sequences of miR-212 on the 3'-UTR of SOX4 mRNA. **B,** MiR-212 mimic reduced the Luciferase activity of the wild-type SOX4 whereas it had no marked change of the mutated SOX4 mRNA. **C,** Upregulation of miR-212 could inhibit the mRNA level of SOX4, while downregulation of miR-212 increased the expression of SOX4 in SW480 cells.

Table I. MiR-212 expression and clinicopathological features in 53 colorectal cancer.

Clinicopathological features	Cases (No. = 53)	miR-212 expression		*p*-value*
		25 High (%)	28 Low (%)	
Age (years)				
≤ 50	22	10 (45.5)	9 (54.5)	0.430
> 50	31	15 (48.4)	16 (51.6)	
Tumor size (mm)				
≤5.0	27	16 (59.3)	11 (40.7)	0.072
>5.0	26	9 (34.6)	17 (65.4)	
TNM stage				
I-II	28	17 (60.7)	11 (39.3)	0.037*
III-IV	25	8 (34.8)	17 (65.8)	
Lymph-node metastasis				
0-2	29	18 (62.1)	11 (37.9)	0.017*
>2	24	7 (29.2)	17 (70.8)	
Metastasis				
Present	25	15 (60.0)	10 (40.0)	0.077
Absent	28	10 (35.7)	18 (64.3)	
SOX4				
Negative	25	16 (64.0)	9 (36.0)	0.020*
Positive	28	9 (32.1)	19 (67.9)	

**p*-values are calculated with the Chi-square test.

play great functions in a variety of tumors, included colorectal cancer[13]. MiR-212 suppressed cell proliferation, migration and invasion by targeting to MAPK1 in prostate cancer and also in renal cell carcinoma[14,15]. Our results were similar to all the findings, and we found that miR-212 was down-regulated in colorectal cancer tissues and CRC cell lines LOVO and SW480 in comparison with the corresponding non-tumor tissues and the colorectal epithelial cell CCD-18Co. In addition, we found that overexpression of miR-212 could inhibit the migration and invasion, while knockdown of miR-212 promoted the migration and invasion of CRC cell line. Moreover, miR-212 acted as a tumor suppressor in thyroid cancer and also inhibited the EMT of triple negative breast cancer[16,17]. Our re-

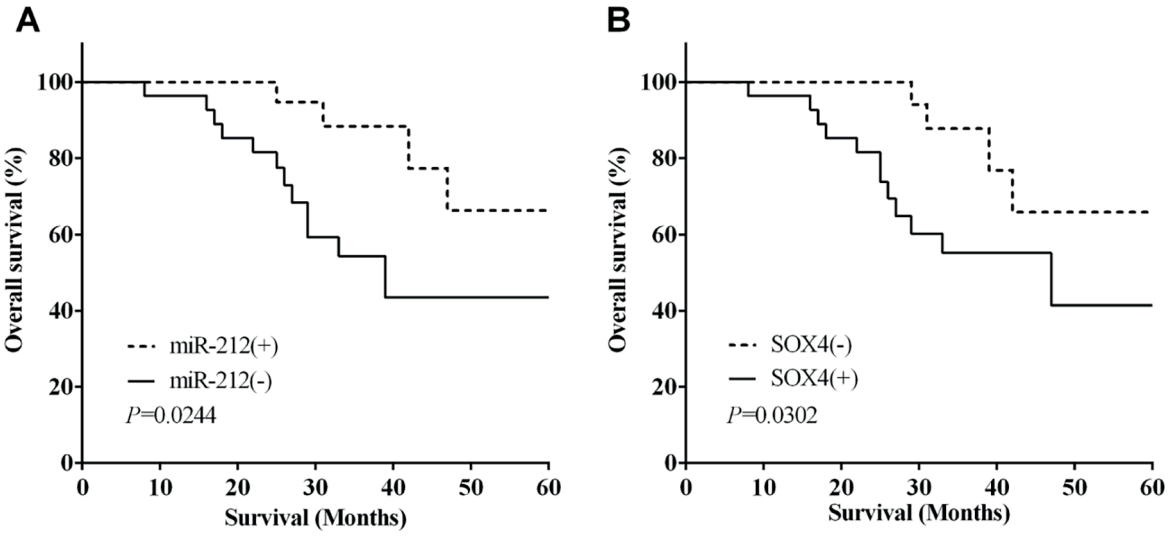

Figure 6. Identification of miR-212 and SOX4 associated with poor survival in colorectal cancer. **A,** The Kaplan-Meier analysis demonstrated that low expression of miR-212 patients had a shorter OS than that of the patients with high expression of miR-212. **B,** Overexpression of SOX4 predicted wore outcome of CRC patients.

sults were consistent with the findings above; miR-212 overexpression suppressed the EMT, whereas inhibited miR-212 could promote the EMT of CRC cells. Additionally, we discovered that downregulation of miR-212 was associated with poor prognosis of colorectal cancer patients, which was consistent with Tang et al[27] in NSLC.

SOX4 acts as a transcriptional regulator and is overexpressed in kinds of tumors including CRC, which usually correlates with cell apoptosis and tumor angiogenesis[18,19]. SOX4 has been found to promote the proliferation, migration and invasion in melanoma cells[20,21]. Consistent with that, we found that SOX4 was upregulated in colorectal cancer tissues and cell lines. Interference of SOX4 could inhibit the migration and invasion in CRC cell line SW480. Furthermore, overexpression of SOX4 promoted the metastasis and induced the EMT in renal cell carcinoma, lung adenocarcinoma and non-small cell lung cancer[22-25]. Our results were consistent with the findings; downregulation of SOX4 could suppress the EMT in CRC cell line SW480. Moreover, we discovered that the expression of miR-212 had an inverse connection with SOX4 expression in colorectal cancer tissues. We first propose that SOX4 was a direct target gene of miR-212 and the expression of SOX4 was mediated by miR-212, which was consistent with the findings of Tang et al[27] and Mehta et al[28]. In osteosarcoma, miR-212 has been reported to inhibit the proliferation and invasion by directly targeting the 3'-UTR of SOX4 mRNA[29]. Similar findings were reported by Fu et al[30] that miR-212 could suppress EMT of prostate cancer cells by directly targeting the 3'-UTR of SOX4 mRNA. Our results were consistent with all the findings showing that miR-212 suppresses the migration, invasion and EMT by directly targeting the 3'-UTR of SOX4 mRNA in CRC cell line. Moreover, we discovered that overexpression of SOX4 could cause shorter overall survival, which is consistent with Lu et al[31].

Conclusions

The expression of miR-212 was lower, while SOX4 overexpression was higher in CRC tissues and cells. Overexpression of miR-212 and knockdown of SOX4 inhibited the migration, invasion and EMT of CRC cell line. Moreover, the expression of miR-212 had a negative connection with the expression of SOX4 and miR-212 directly targeted to the 3'-UTR of SOX4 mRNA. MiR-212 mediated the expression of SOX4 by directly binding to the 3'-UTR of SOX4 mRNA. In addition, low expression of miR-212 or overexpression of SOX4 predicted poor prognosis in colorectal cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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