

MicroRNA-432-5p inhibits cell migration and invasion by targeting CXCL5 in colorectal cancer

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Abstract. MicroRNAs (miRNAs) play an important role in the occurrence and development of colorectal cancer (CRC). Evidence shows that miR-432-5p expression is decreased in various tumors and cancer cell lines. miR-432-5p can inhibit tumor invasion and metastasis, but its role in colorectal cancer is unclear. The present study demonstrated that miR-432-5p expression was decreased in colorectal cancer tissue and cell lines, and is negatively associated with invasion classification, lymph node metastasis and Tumor-Node-Metastasis stage. Kaplan-Meier survival analysis showed that low miR-432-5p expression was associated with a poor survival rate in patients with CRC. In addition, SW480 and HT-29 cells transfected with miR-432-5p mimics had decreased migration and invasion abilities, whereas miR-432-5p inhibitors had the opposite effect. The expression of C-X-C motif chemokine ligand 5 (CXCL5), a direct target of miR-432-5p, was negatively associated with miR-432-5p expression. When CXCL5 was introduced into miR-432-5p mimic-transfected SW480 and HT-29 cells, miR-432-5p-mediated inhibition of CRC migration and invasion was reversed. Thus, the present results suggest that miR-432-5p can inhibit the migration and invasion of CRC cells by targeting CXCL5.

Introduction

Colorectal cancer (CRC), one of the most common cancer types of the digestive system, is the fourth leading cause of cancer-associated deaths worldwide, resulting in ~900,000 deaths annually (1). New technologies for the diagnosis (such as computerized tomography and enteroscopy) and treatment (endoscopic and surgical resection, chemotherapy, targeted treatment and immunotherapy) of CRC have doubled the overall survival rate of patients; however, recurrence and metastasis still contribute towards poor prognosis (2). Studies have shown that 30-50% of patients with CRC experience cancer recurrence and/or metastasis, of which distant liver metastasis is the most common (1,2).

The molecular mechanism underlying colorectal cancer metastasis is complex; it is a multi-step and multi-factor biological process, including oncogene activation and tumor suppressor gene inactivation (3). Although genetic and epigenetic alterations underpin the development of CRC, the molecular mechanisms underlying neoplastic progression remain unclear. Therefore, a comprehensive understanding of the molecular mechanism of cancer recurrence and metastasis, and the identification of target sites for intervention, is required for the evaluation of prognosis and the development of treatments for patients with CRC.

MicroRNAs (miRNAs/miRs) are 19-24 nucleotide-long, noncoding RNA molecules, that bind to the 3'-untranslated region (3'-UTR) of genes to negatively regulate their expression (4). Dysregulated miRNAs may play crucial roles in CRC initiation and progression through their improper regulation of various biological functions, depending on their target genes (5). miRNAs are not only involved in the proliferation, invasion, metastasis and angiogenesis of CRC cells, but are also closely associated with the clinical stage and prognosis of CRC (6). In CRC, miRNAs regulate the expression of oncogenes and tumor suppressor genes, regulating the interstitial transformation and adhesion of colon epithelial cells, the methylation of colon DNA, and angiogenesis (7). Therefore, research on miRNAs in CRC is of great significance for the early diagnosis, treatment and prognosis of patients with colorectal cancer.

miR-432-5p is an important tumor suppressor molecule that was shown to be significantly downregulated in various

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Abbreviations: CRC, colorectal carcinoma; CXCL5, C-X-C motif chemokine ligand 5; miRNA/miR, microRNA

Key words: colorectal cancer, miR-432-5p, migration, invasion, CXCL5

tumors, including ovarian cancer (8), cervical cancer (9,10), pituitary adenomas (11), hepatocellular carcinoma (12), osteosarcoma (13), lung adenocarcinoma (14) and nasopharyngeal carcinoma (15). However, the expression, function, and potential molecular mechanisms of miR-432-5p in CRC are still unclear. The purpose of the present study was to evaluate the expression of miR-432-5p and its clinical significance in CRC, and to examine the potential molecular mechanism of miR-432-5p in cancer progression.

Materials and methods

Tissue samples and ethics statement. A total of 37 paired CRC tissue samples and corresponding adjacent tissue samples (at least 5 cm from the edge of the tumor) were obtained from the Wuhan No. 1 Hospital (Wuhan, China) between August 2014 and April 2015. The patients (20 males and 17 females; mean age, 53.62±11.75 years) did not receive radiotherapy or chemotherapy before the operation and the final diagnosis was based on postoperative pathological diagnosis. This study was approved by the Wuhan No. 1 Hospital Ethics Committee, and all patients signed the informed consent form.

Cell culture. Four human colorectal cancer cell lines, SW480, SW620, HT-29 and HCT116 were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The human colon epithelium cell line, NCM460, was provided by Dr. Kong (Department of Oncology, Puai Hospital, Huazhong University of Science and Technology, China). Cells were cultured in RPMI-1640 medium (HyClone; Cytvia) supplemented with 10% FBS (HyClone; Cytvia) and 1% penicillin-streptomycin (Beyotime Institute of Biotechnology) at 37°C in an atmosphere with 5% CO₂. Cell line authentication was achieved by genetic profiling using the short tandem repeat (STR) method at the Tongji Medical College.

Plasmids and transfection. The miR-432-5p mimic (5'-UCU UGGAGUAGGUCAUUGGGUGG-3'), mimic negative control (5'-CGAUCGCAUCAGCAUCGAUUGC-3'), miR-432-5p inhibitor (5'-CCACCCAAUHACCUACUCCAAGA-3'), and inhibitor negative control (5'-UGAGCUGCAUAGAGUAGU GAUUA-3') were purchased from Guangzhou RiboBio Co., Ltd. The C-X-C motif chemokine ligand 5 (CXCL5) overexpression plasmid (pcDNA3.1-CXCL5) and its negative control (pcDNA3.1) were purchased from BioMedical Co., Ltd. Approximately 3x10⁵ cells were seeded into 6-well plates and grown to 70% confluence. Then, miR-432-5p mimic (50 nM), miR-432-5p inhibitor (100 nM), mimic NC (50 nM), or inhibitor NC (100 nM) were transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). For the phenotype reversal experiment, pcDNA3.1-CXCL5 (50 ng) or pcDNA3.1 plasmid (50 ng) were co-transfected with miR-432-5p mimic (50 nM) or mimic NC (50 nM) into cells using Lipofectamine 2000. After 8 h, the medium was replaced with fresh medium. After 48 h, the cells were collected for subsequent experiments

Quantitative analysis of miRNAs and mRNAs. The total RNA from the tissue samples and cells was extracted using the TRIzol[®] method (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA was then reverse transcribed into cDNA using the

RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The reverse transcription reaction conditions were as follows: 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. Real-time quantitative PCR was used for amplification. The PCR conditions were as follows: 95°C for 10 min, 95°C for 30 sec, 95°C for 15 sec, 62°C for 30 sec, for 35 cycles in total. The PCR reaction ended at 4°C. U6 RNA or GAPDH were used as internal references. The data of three independent samples, after three independent experiments, were analyzed using the 2^{-ΔΔC_q} method (16). Primer sequences used were as follows: miR-432-5p forward, 5'-AACGAGACGACG ACAGACT-3'; miR-432-5p reverse, 5'-CTTGGAGTAGGT CATTGGGT-3'; CXCL5 forward, 5'-TGGACGGTGGAA ACAAGG-3'; CXCL5 reverse, 5'-CTTCCCTGGGTTTCAG AGAC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3'; U6 reverse, 5'-CGCTTCACGAATTTGCGTGTGCAT-3'; GAPDH forward, 5'-GGTGAAGGTCGGAGTCAACG-3'; and GAPDH reverse, 5'-CAAAGTTGTCATGGATGHACC-3'.

Wound-healing assay. For migration analysis, ~2x10⁵ cells were plated into 12-well plates and cultured until 100% confluence. A 10-μl micropipette tip was used to scratch the cell monolayer. After washing with pre-warmed PBS, the cells were incubated with RPMI-1640 medium containing 1% FBS. Cell migration towards the scratch was observed under an inverted light microscope (magnification, x40) for 48 h and images were captured. The width of three scratches with equal spacing along the scratch edge were measured, and the average value was calculated. The following formula was used for calculations of wound healing: Scratch healing rate (%)=(0 h scratch width-48 h scratch width)/0 h scratch width x100%.

Transwell assay. At 48 h following transfection, cells were digested and resuspended in FBS-free RPMI-1640 culture medium and the cell concentration was adjusted to 3x10⁵/ml. Subsequently, a 150 μl cell suspension was added to the upper chambers of the 24-well Transwell with or without Matrigel coating (BD Biosciences), and 500 μl RPMI-1640 medium containing 10% FBS was added to the lower chamber as a chemoattractant. After 12 h of incubation, the noninvasive cells were wiped off with cotton swabs and the wells were washed thrice with PBS. The invasive cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and stained with 0.1% crystal violet at room temperature. In each well, five fields were randomly selected to count the number of cells using a light microscope (magnification, x100).

Bioinformatics analysis and dual luciferase reporter assay. TargetScan (http://www.targetscan.org/mamm_31/), miRcode (<http://www.mircode.org/>) and miRDB (<http://mirdb.org/>) databases were used to predict the target genes of miR-432-5p. A CXCL5 3'-UTR wild-type or mutant sequence was inserted into the pGL3 promoter vector (Invitrogen; Thermo Fisher Scientific, Inc.), which was defined as pGL3-CXCL5-WT or pGL3-CXCL5-MUT, respectively. The 293T cells were seeded into 24-well plates (5x10³/well) and cultured for 24 h. The pGL3-CXCL5-WT (50 ng) or pGL3-CXCL5-MUT reporter plasmids (50 ng) were co-transfected with miR-432-5p mimic (50 nM) or mimic NC (50 nM) into cells using Lipofectamine

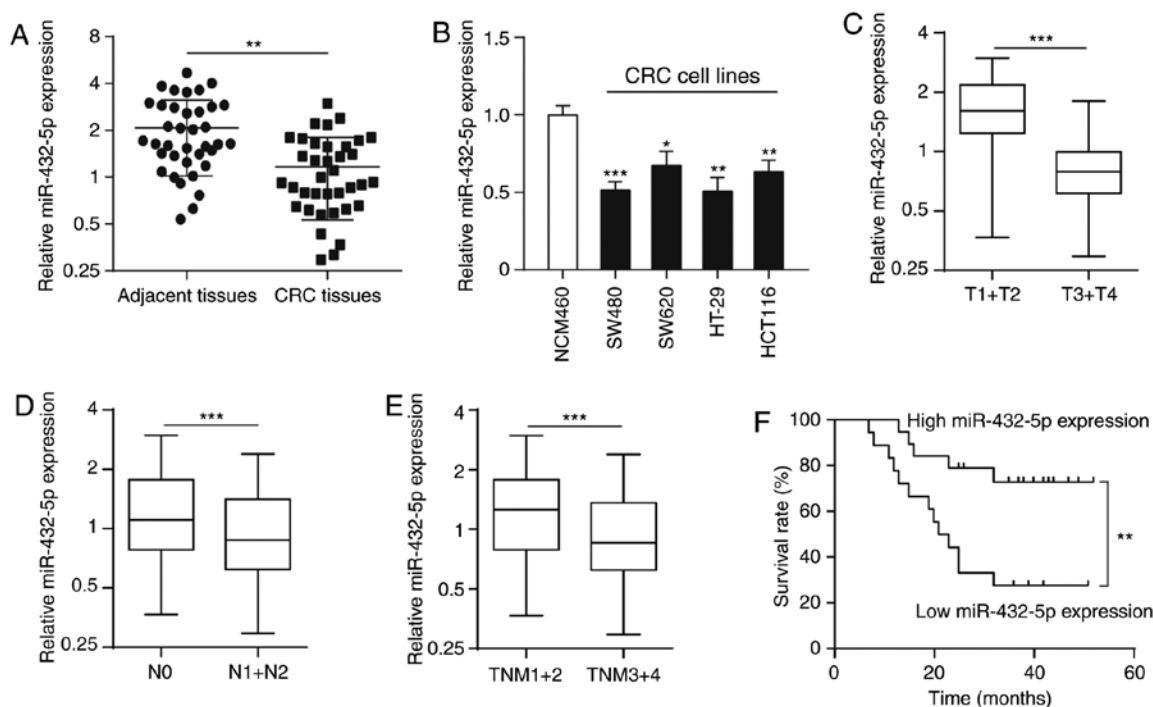


Figure 1. Low miR-432-5p expression is associated with clinicopathological factors in patients with CRC. (A) miR-432-5p expression is lower in CRC tissues compared with adjacent tissues, detected by reverse transcription-quantitative PCR analysis. (B) miR-432-5p expression was significantly decreased in four CRC cell lines (SW480, SW620, HT-29 and HCT116) compared with the human colon epithelium cell line NCM460. (C-E) miR-432-5p expression was significantly decreased in patients with CRC showing high invasion, lymph node metastasis and TNM stage. (F) Kaplan-Meier survival curves for CRC patients with high or low miR-432-5p expression level. Low miR-432-5p expression was significantly associated with poor overall survival in patients with CRC. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. CRC, colorectal cancer; miR, microRNA; TNM, Tumor-Node-Metastasis.

2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h after transfection, luciferase signals were measured using the Dual Luciferase Reporter Assay kit (Promega Corporation).

Western blot analysis. Total protein was extracted from cells using RIPA lysis (Thermo Fisher Scientific, Inc.) and extraction buffer with a protease inhibitor cocktail at 4°C. Protein concentrations were determined using the BCA protein concentration test kit (Beyotime Institute of Biotechnology). Approximately 50 µg protein from each sample was denatured and separated by 10% SDS-PAGE and transferred to PVDF membranes by the conventional wet transfer method. Then, the membrane was incubated in 5% skim milk powder at room temperature for 2 h, followed by incubation with primary antibodies against CXCL5 (1:2,000; cat. no. ab126763) and β-actin (1:2,000; cat. no. ab8227; both from Abcam) overnight at 4°C. After washing with TBST buffer, HRP-conjugated goat anti-rabbit secondary antibodies (1:3,000, cat. no. ab205718; Abcam) were added and the membrane was incubated at room temperature for 2 h, followed by ECL chemiluminescence detection.

Statistical analysis. SPSS 20.0 statistical software (SPSS, Inc.) was used for data statistics and analysis. Data are represented as mean ± standard deviation in line with the normal distribution. Student's t-test (paired or unpaired) was used to analyze differences between two groups. One-way ANOVA, followed by Tukey's multiple comparison test, was used to analyze the differences among multiple groups. Spearman's correlation analysis was used to analyze the association

between miR-432-5p and CXCL5 expression. The prognostic significance of miR-432-5p was assessed using a Kaplan-Meier survival curve and groups were compared using the log-rank test. $P < 0.05$ was considered to indicate as statistically significant difference.

Results

miR-432-5p expression is decreased in CRC tissue and cell lines. Tumor tissues were collected from 37 patients with CRC, as well as their corresponding adjacent tissues, and measured the expression level of miR-432-5p by reverse transcription qPCR. The results showed that the miR-432-5p expression was significantly lower in CRC tissues compared with adjacent tissues (Fig. 1A). In addition, compared with the human colon epithelium cell line (NCM460), the expression of miR-432-5p in CRC cell lines (SW480, SW620, HT-29 and HCT116) was significantly lower (Fig. 1B).

Low expression of miR-432-5p is associated with clinicopathological factors in CRC. In order to evaluate the clinical significance of miR-432-5p, the association between miR-432-5p expression and clinicopathological factors were evaluated. Interestingly, miR-432-5p expression was significantly associated with invasion classification (Fig. 1C), lymph node metastasis (Fig. 1D) and Tumor-Node-Metastasis stage (Fig. 1E) in CRC samples. The 37 patients with CRC were classified into either an miR-432-5p low (n=18) or high expression groups (n=19) using the median expression level of miR-432-5p (0.926)

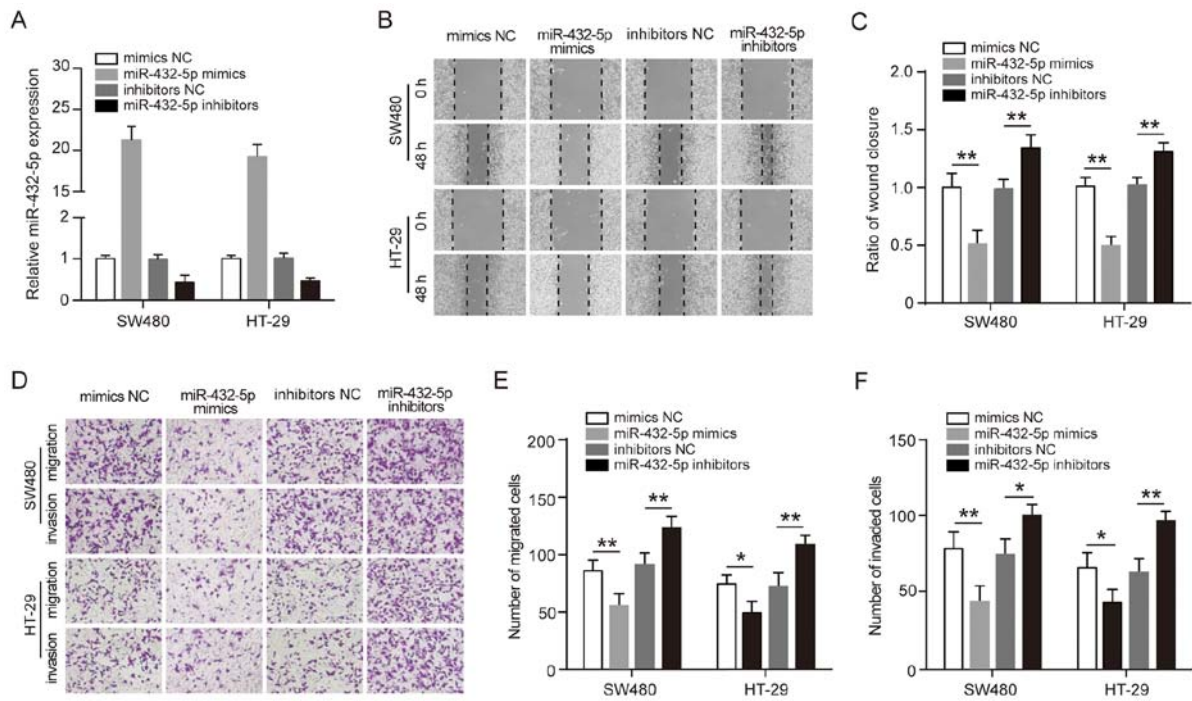


Figure 2. miR-432-5p inhibits the invasion and migration of SW480 and HT-29 cells. (A) The efficiency of the miR-432-5p mimics or inhibitors in CRC cells was detected by reverse transcription-quantitative PCR. (B and C) Wound healing assay was performed to investigate the effect of miR-432-5p mimics or inhibitors on SW480 and HT-29 cell migration. (D-F) Transwell assay was performed to investigate the effect of miR-432-5p mimics or inhibitors on SW480 and HT-29 cell migration and invasion. Bar, 200 μ m. * P <0.05; ** P <0.01. miR, microRNA; CRC, colorectal cancer; NC, negative control.

as a cutoff value. Kaplan-Meier survival analysis showed that patients in the miR-432-5p low expression group had a significantly shorter overall survival rate compared with those in the high expression group (Fig. 1F).

miR-432-5p inhibits migration and invasion of CRC cells. The association between low miR-432-5p expression and pathological factors and prognosis of CRC suggests that miR-432-5p may play an anti-tumor role. To study the potential anti-tumor mechanism of miR-432-5p, SW480 and HT-29 cells were transfected with miR-432-5p mimics or inhibitors to observe the effect of miR-432-5p on cell migration and invasion (Fig. 2A). The wound healing assay showed that miR-432-5p mimics significantly inhibited the mobility of SW480 and HT-29 cells, whereas, miR-432-5p inhibitors significantly promoted the mobility of the cells (Fig. 2B-C). In addition, Transwell assay was performed to investigate the effect of miR-432-5p on CRC cells migration and invasion. It was revealed that the number of cells transfected with miR-432-5p mimics was significantly decreased, but the number of cells transfected with miR-432-5p inhibitors was significantly increased (Fig. 2D-F). These results suggest that miR-432-5p may have anti-migration and anti-invasive roles in CRC cells.

CXCL5 is a direct target gene of miR-432-5p. To investigate the mechanisms of miR-432-5p in CRC, the target genes of miR-432-5p were analyzed using three online software programs (TargetScan, miRcode and miRDB). Based on bioinformatics analysis results, it was revealed that CXCL5, which is an important protein associated with tumor migration and invasion, contained the conserved

putative miR-432-5p target site (Fig. 3A). A dual-luciferase reporter analysis showed that miR-432-5p mimics significantly inhibited the reporter activity of the CXCL5 3'-UTR wild-type reporter plasmid but not the mutant reporter plasmid (Fig. 3B). Following transfection with miR-432-5p mimics, endogenous CXCL5 mRNA and protein expression was significantly decreased in SW480 and HT-29 cells. In contrast the CXCL5 mRNA and protein expression was significantly increased in cells treated with miR-432-5p inhibitor (Fig. 3C-D). Furthermore, it was found that the CXCL5 mRNA expression levels were significantly upregulated in CRC tissues compared with corresponding adjacent tissues (Fig. 3E). A significant negative correlation was also observed between the miR-432-5p levels and CXCL5 mRNA levels in CRC tissues (Fig. 3F).

CXCL5 reverses the inhibitory effects of miR-432-5p on the malignant phenotypes of CRC cells. To further evaluate whether miR-432-5p inhibits migration and invasion by specifically targeting CXCL5, pcDNA3.1-CXCL5 plasmid was reintroduced into miR-432-5p mimic-transfected SW480 or HT-29 cells, and western blot analysis was performed for CXCL5 protein expression (Fig. 4A). Wound healing assays showed that overexpression of CXCL5 reversed the inhibitory effect of miR-432-5p on the mobility of SW480 or HT-29 cells (Fig. 4B and C). Similarly, the transwell assay showed that overexpression of CXCL5 reversed the inhibitory effect of miR-432-5p on the migration and invasion of SW480 or HT-29 cells (Fig. 4D-F). These results indicated that miR-432-5p could inhibit tumor cell migration and invasion by directly targeting CXCL5 in CRC.

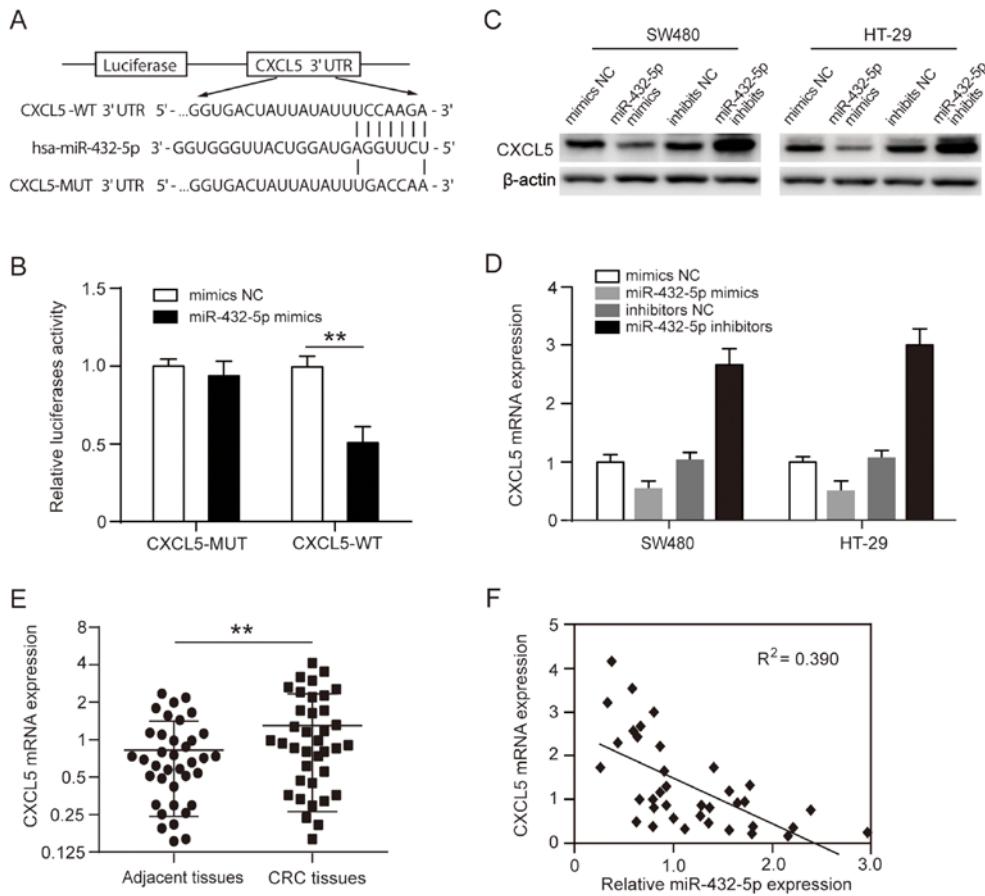


Figure 3. CXCL5 is a target of miR-432-5p in CRC cells. (A) CXCL5 3'-UTR wild-type and mutant binding sites for miR-432-5p. (B) Relative luciferase activity results in 293T cells after co-transfection with miR-432-5p mimics and wild-type or mutant CXCL5 3'-UTR plasmid. (C and D) CXCL5 mRNA and protein expression was detected in SW480 and HT-29 cells after transfection with miR-432-5p mimics or inhibitors. (E) CXCL5 mRNA expression was higher in CRC tissues compared with adjacent tissues using reverse transcription-quantitative PCR analysis. (F) CXCL5 mRNA expression was negatively correlated with miR-432-5p expression in CRC tissues by Spearman's correlation analysis. * $P < 0.05$. CXCL5, C-X-C motif chemokine ligand 5; miR, microRNA; CRC, colorectal cancer; UTR, untranslated region; MUT, mutant; WT, wild type; NC, negative control.

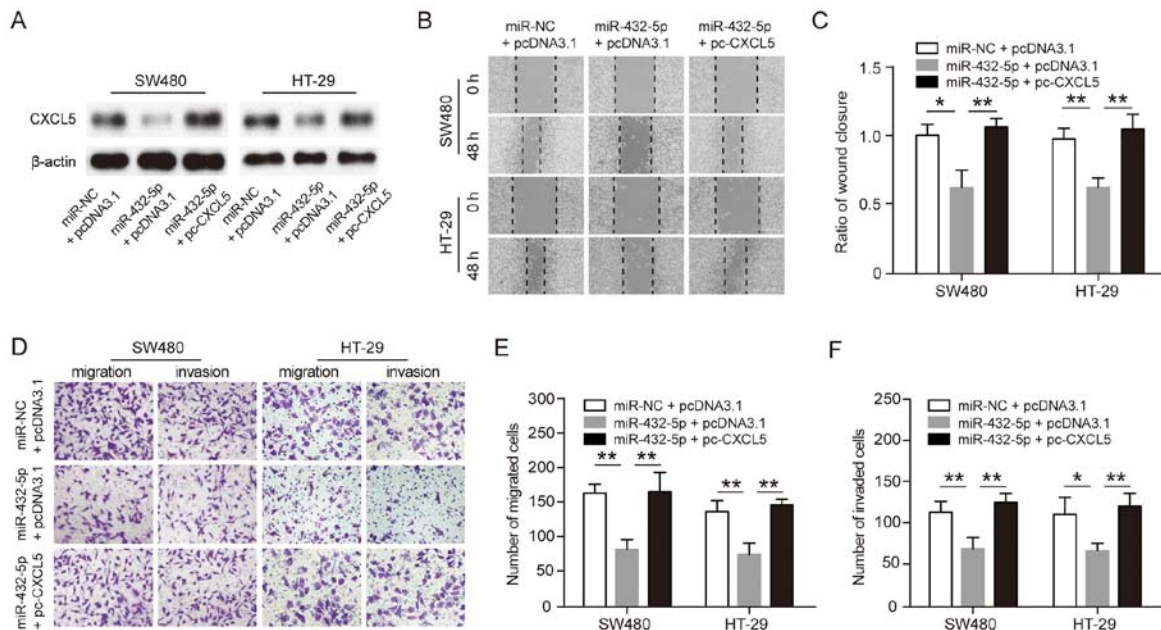


Figure 4. CXCL5 reverses the inhibitory effects of miR-432-5p on colorectal cancer cell invasion and migration of SW480 and HT-29 cells. (A) CXCL5 protein expression was detected in SW480 and HT-29 cells after co-transfection of miR-432-5p mimics and pcDNA3.1-CXCL5 (pc-CXCL5) plasmids. (B and C) The effect of CXCL5 on SW480 and HT-29 cell migration as determined by the wound healing assay. (D-F) The effect of CXCL5 on SW480 and HT-29 cell migration and invasion determined by transwell assay. Bar, 200 μm . * $P < 0.05$; ** $P < 0.01$. CXCL5, C-X-C motif chemokine ligand 5; miR, microRNA; NC, negative control.

Discussion

miRNAs have been shown to regulate the expression of tumor-associated genes, resulting in the inhibition or promotion of the proliferation, differentiation, invasion and metastasis of tumor cells. Therefore, elucidating the potential mechanism of miRNAs in tumor progression may aid in tumor diagnosis and treatment (17,18). miR-432-5p has been reported to be expressed at low levels in many types of tumors and could play a role in tumor development. For example, the expression of miR-432-5p is significantly decreased in hepatocellular carcinoma, and overexpression of miR-432-5p inhibits the proliferation and tumorigenicity of tumor cells through its regulation of the Wnt/ β -catenin signaling pathway (12). Overexpression of miR-432-5p can block the G₀-G₁ phase cells of neuroblastoma and decrease cell proliferation; thus, miR-432-5p could have an important role in the progression of neuroblastoma (19). In addition, overexpression of miR-432-5p not only inhibits the proliferation of lung cancer cells, but also enhances their sensitivity to cisplatin treatment (14). However, miR-432-5p is abnormally highly expressed in metastatic melanomas (20). Therefore, it remains unclear whether miR-432-5p targets tumor suppressor genes or oncogenes. It is speculated that the expression and function of miR-432-5p may be tissue- and tumor-dependent.

In the present study, it was found that the expression of miR-432-5p was significantly decreased in human CRC tissues and cell lines, and that low miR-432-5p expression was associated with advanced tumor stages. Importantly, low miR-432-5p expression is associated with poor overall survival; therefore, miR-432-5p expression may provide important information on tumor progression in patients with CRC. Further functional analysis demonstrated that miR-432-5p significantly inhibited CRC cell migration and invasion *in vitro*. In order to determine which target genes are regulated by miR-432-5p to inhibit the migration and invasion of CRC cells, a bioinformatic prediction analysis was carried out and selected several target genes that were closely associated with the migration and invasion function, for experimental verification. This analysis identified CXCL5 as a putative target of miR-432-5p.

CXCL5, also known as neutrophil activating peptide 78, is located on human chromosome 4q13-q21, and is an important chemokine in the tumor microenvironment (21). Studies have shown that CXCL5 is highly expressed in different malignant tumor types, such as lung cancer, breast cancer, pancreatic cancer and non-small cell lung cancer (22). The high expression of CXCL5 is not only derived from primary tumor cells, but is also secreted by immune cells in the tumor microenvironment, including macrophages, neutrophils and T/B lymphocytes (22). During tumor progression, CXCL5 binds to its receptor, CXCR2, to recruit immune cells and promote tumor growth, invasion, metastasis and angiogenesis (22). In addition, the overexpression of CXCL5 is closely associated with survival, recurrence and metastasis in cancer patients (23). In CRC, the expression of CXCL5 in metastatic tissues is significantly higher compared with the adjacent tissues (24,25). Moreover, the expression of CXCL5 is positively associated with the degree of inflammatory infiltration, malignancy, metastatic potential, and poor prognosis in colorectal cancer (26,27). In the present study, CXCL5 was

shown to be a target of miR-432-5p as its expression in tumor tissues was negatively correlated with miR-432-5p expression. When CXCL5 was introduced into SW480 and HT-29 cells transfected with an miR-432-5p mimic, the inhibitory effect of miR-432-5p on CRC migration and invasion was reversed. In conclusion, the results suggest that miR-432-5p can inhibit the migration and invasion of CRC cells by targeting CXCL5.

In conclusion, the present study revealed that miR-432-5p is downregulated in CRC tissues and in various CRC cell lines, and its low expression levels were associated with clinicopathological features of patients with CRC. Furthermore, overexpression of miR-432-5p in CRC cell lines decreased cell migration and invasion, whereas miR-432-5p inhibitors had the opposite effect. CXCL5 was identified as a direct target of miR-432-5p and demonstrated that miR-432-5p functions as a tumor suppressor by negatively regulating CXCL5 expression.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ML and LL conceived and designed the project; ML, ZH and LL acquired the data; ML, ZH, and YK analyzed and interpreted the data; and all authors wrote the paper. ML and LL confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was performed in accordance with the Declaration of Helsinki and approved by the Wuhan No. 1 Hospital Ethics Committee (Wuhan, China; approval no. WH201403001) and written informed consent was obtained from each study participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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