

# miR-7-5p regulates the proliferation and migration of colorectal cancer cells by negatively regulating the expression of Krüppel-like factor 4

MINGJUN DONG, YANGYANG XIE and YIDONG XU

Department of Anorectal Surgery, The No. 2 Hospital of Ningbo, Ningbo, Zhejiang 315010, P.R. China

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**Abstract.** Previous studies have demonstrated that microRNA-7-5p (miR-7-5p) functions as a tumor suppressor in certain types of human cancer. However, the role of miR-7-5p in colorectal cancer (CRC) remains to be investigated. Using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), the present study demonstrated that miR-7-5p was downregulated in CRC tissues and cell lines. In addition, low miR-7-5p expression is able to predict a poor 5-year overall survival rate for patients with CRC. *In vitro* studies revealed that miR-7-5p overexpression inhibits cell proliferation and migration. Furthermore, Krüppel-like factor 4 (KLF4), an oncogene in CRC, was validated as a direct target of miR-7-5p. KLF4 expression was negatively correlated with miR-7-5p expression in CRC tissues. Notably, KLF4 overexpression rescued the suppressive effects of miR-7-5p on CRC cell proliferation and migration. In summary, the results of this study demonstrated that miR-7-5p inhibits CRC proliferation and migration by targeting KLF4, which suggests that miR-7-5p is a potential molecular target for the treatment of human CRC.

## Introduction

The incidence and mortality rates of colorectal cancer (CRC) in the USA have declined between 1975 and 2006 due to decreased smoking and red meat consumption (1). However, both the incidence and mortality rates of CRC have rapidly increased in Asian countries in the past three decades (2,3). Currently, CRC is the third most common type of cancer worldwide, highlighting the importance of developing novel anticancer methods (4).

MicroRNAs (miRs) are a family of non-coding RNAs that can regulate gene expression predominantly by directly

binding to the 3'-untranslated region (3'-UTR) of target mRNAs (5). It is estimated that up to 33% of human genes can be regulated by miRs (6). Previously, the importance of miRs in regulating functions of malignant cells, including cell proliferation, migration, invasion and apoptosis, has been appreciated (7,8). Altered expression levels of miRs have been identified in multiple types of human tumor, including CRC, and miRs have been recognized as biomarkers for tumor diagnosis and treatment (9-11).

miR-7-5p has been recognized as a tumor suppressor in melanoma and breast cancer, as it is frequently downregulated in these tumor types (12-14). Giles *et al* (12) reported that miR-7-5p expression was reduced in metastatic melanoma-derived cells compared with primary melanoma cells, and its effects on melanoma cell migration and invasion was exerted partly via inhibition of insulin receptor substrate 2 expression and oncogenic Akt signaling. In addition, it has been identified that miR-7-5p is a potent inhibitor of melanoma growth and metastasis by inactivation of the transcription factor p65/nuclear factor- $\kappa$ B signaling pathway, which suggests that miR-7-5p may serve a role in therapy for this disease (13). Furthermore, *in vitro* and *in vivo* studies revealed that miR-7-5p overexpression could inhibit breast cancer cell proliferation and induce cell apoptosis by targeting REG $\gamma$  (14). However, to the best of our knowledge, the underlying mechanisms of miR-7-5p in CRC progression remain unknown.

Krüppel-like factor 4 (KLF4) has been reported to serve a critical role in cell differentiation and development (15). Evidence has demonstrated that KLF4 can function as either a tumor suppressor or an oncogene in human tumors (16,17). Previous studies revealed that KLF4 expression was upregulated in CRC and could be regulated by miRs, including miR-92a and miR-543 (18,19). Given the importance of miR-7-5p and KLF4 in tumor initiation and development, the current study investigated whether miR-7-5p could regulate KLF4 in CRC. Furthermore, the effects of miR-7-5p and KLF4 expression levels on cell proliferation and migration were examined.

## Materials and methods

*Patients and tumor tissues.* Human CRC tumor tissues and adjacent non-tumor tissues were obtained from 76 enrolled

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*Correspondence to:* Dr Yidong Xu, Department of Anorectal Surgery, The No. 2 Hospital of Ningbo, 41 Northwest Street, Haishu, Ningbo, Zhejiang 315010, P.R. China  
E-mail: xuyidong\_nbh@126.com

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patients who received surgical treatment between August 2009 and December 2011 at The No. 2 Hospital of Ningbo (Ningbo, China). All patients did not receive any anticancer treatments prior to surgery. The tissue samples were snap-frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until further use. The current study was approved by the Ethics Committee of The No. 2 Hospital of Ningbo (Ningbo, China). Written informed consent was obtained from all enrolled patients. The clinicopathological features were collected and summarized in Table I.

**Cell culture.** The human colon epithelial cell line HCEC ICT and the CRC cell lines HCT-116, SW480 and SW620 were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100  $\mu\text{g}/\text{ml}$  streptomycin and 100 U/ml penicillin (Beyotime Institute of Biotechnology, Haimen, China) in a  $37^{\circ}\text{C}$  humidified incubator containing 5%  $\text{CO}_2$ .

**Cell transfection.** The synthesized miR-7-5p mimic (5'-CAA CAAUCACAGUCUGCCAUA-3'), inhibitor (5'-UAU GGCAGACUGUGAUUUGUUG-3') and negative control miR (NC-miR; 5'-ACCGCUAAUCAUACGAAUACAC-3') were purchased from Chang Jing Bio-Tech, Ltd. (Changsha, China). KLF4 small interfering (si)RNA (si-KLF4; 5'-CCA GCCAGAAAGCACUACAAU-3') and NC siRNA (NC-siR; 5'-AUGCAAUACCGCAGAACACCA-3') were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The KLF4 plasmid, obtained from OriGene Technologies, Inc. (Beijing, China), was also transfected into cells with or without miR-7-5p mimic co-transfection. Transfection was conducted using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) with 50 nM miRNA, 50 nM siRNA, or 1  $\mu\text{g}$  KLF4 plasmid, according to the manufacturer's protocol. Cells were collected 48 h after transfection for subsequent experiments.

**Bioinformatics analysis.** The TargetScan prediction software (version 7.1; [http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)) was used to predict the miRs that could bind to the 3'-UTR of KLF4.

**Luciferase activity assay.** The wild-type (wt) or mutant (Mut) KLF4 3'-UTR was inserted downstream of the firefly luciferase gene within a pmirGLO plasmid (Promega Cooperation, Madison, WI, USA). CRC cells were co-transfected with miR-7-5p mimic or NC-miR and wt or Mut KLF4 3'-UTR using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Relative luciferase activities were measured using a Dual-Luciferase Reporter system (Promega Cooperation) following transfection for 48 h, with firefly luciferase activity used as the internal control.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from tissue samples and cell lines using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A MicroRNA First-Strand Synthesis kit and a SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II kit (both from Takara

Biotechnology Co., Ltd., Dalian, China) were employed to quantify the expression levels of miR-7-5p, according to the manufacturer's protocols. The following thermocycling conditions were used: 10 min at  $95^{\circ}\text{C}$ ; 40 cycles of 1 min at  $95^{\circ}\text{C}$ ; 2 min at  $63^{\circ}\text{C}$ ; and 1 min at  $72^{\circ}\text{C}$ . U6 small nuclear (sn)RNA was used as an endogenous control. The comparative cycle threshold ( $2^{-\Delta\Delta\text{C}_q}$ ) method was used to quantify the expression levels of miR-7-5p (20). The primers used were as follows: miR-7-5p forward, 5'-ACA CTCCAGCTGGGTGGAAGACTAGTGATTT-3' and reverse, 5'-CTCAACTGGTGTCTGGAGTCGGCAAT TCAGTTGAGACAACAAA-3'; and U6 snRNA forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCT TCACGAATTTGCGT-3'.

**Western blot analysis.** Total protein was extracted from the tissue samples and cell lines using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). Protein concentration was quantified using an Enhanced BCA Protein assay kit (Beyotime Institute of Biotechnology). Subsequently, the proteins (50  $\mu\text{g}$ ) were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Following blocking with 5% fat-free milk at  $4^{\circ}\text{C}$  for 4 h, the membranes were incubated with the primary antibodies rabbit monoclonal anti-KLF4 (1:1,000; catalog no. ab215036) and rabbit monoclonal anti- $\beta$ -actin (1:1,000; catalog no. ab115777; both from Abcam, Cambridge, UK) overnight at  $4^{\circ}\text{C}$ . Following washing with TBS and Tween-20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:3,000; catalog no. ab205718; Abcam) for 1 h at room temperature. The signals were developed with an enhanced chemiluminescent kit (Beyotime Institute of Biotechnology) and analyzed using ImageJ v.1.42 software (National Institutes of Health, Bethesda, MD, USA).

**Cell proliferation assay.** Cell proliferation rate was analyzed using Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Briefly, the cells were seeded in 24-well plates at a density of  $5 \times 10^3$  cells/well. Subsequently, 10  $\mu\text{l}$  CCK-8 solution was added to each well at 0, 24, 48 and 72 h, and the cells were further incubated for 4 h at  $37^{\circ}\text{C}$ . The optical density was measured at 450 nm.

**Cell migration assay.** Cell migration rate was analyzed using a wound healing assay. The cells were seeded in 6-well plates and cultured until 80% confluence. Subsequently, a wound in the cell surface was created using a 100- $\mu\text{l}$  sterilized pipette tip. At 0 and 24 h after the wound was created, images were obtained using a light microscope (magnification, x200) and analyzed using ImageJ v.1.42 software (National Institutes of Health).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. All statistical analysis was conducted using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were analyzed using analysis of variance followed by Tukey's test or the Student's t-test. Associations between miR-7-5p and clinicopathological

Table I. Associations of miR-7-5p expression with the clinicopathological features of colorectal cancer.

Variable	No. of cases	microRNA-7-5p expression, n		P-value
		High	Low	
Sex				0.381
Male	42	15	27	
Female	37	14	23	
Age, years				0.629
$\geq 50$	40	14	26	
$< 50$	39	15	24	
Tumor size, cm				0.024
$\geq 5$	46	17	29	
$< 5$	33	12	21	
Tumor stage				0.014
I-II	33	11	22	
III-IV	46	18	28	

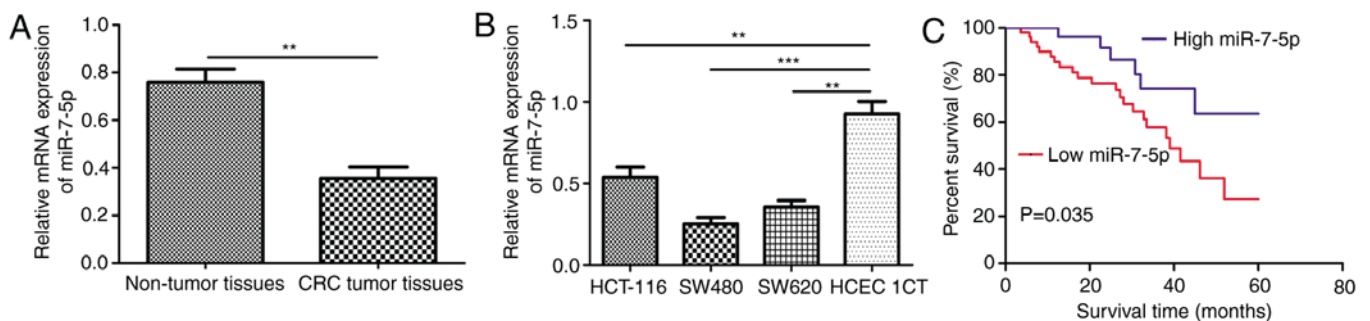


Figure 1. Downregulation of miR-7-5p is associated with overall survival. (A) miR-7-5p expression levels in 79 pairs of CRC tumor tissues and non-tumor tissues were analyzed by RT-qPCR. (B) miR-7-5p expression levels in the human colon epithelial cell line (HCEC 1CT) and CRC cell lines (HCT-116, SW480 and SW620) were analyzed by RT-qPCR. (C) The association between miR-7-5p expression and overall survival of patients with CRC. Data are presented as the mean  $\pm$  standard deviation. \*\*P<0.01; \*\*\*P<0.001. miR-7-5p, microRNA-7-5p; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CRC, colorectal cancer.

features were analyzed by Chi-square test. Correlation between miR-7-5p and KLF4 expression levels was analyzed by Spearman's correlation coefficient. Overall survival was analyzed by a Kaplan-Meier curve and log-rank test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**miR-7-5p expression is reduced in CRC tissues and cell lines.** RT-qPCR demonstrated a significantly lower miR-7-5p expression level in CRC tumor tissues compared with adjacent non-tumor tissues (Fig. 1A). Furthermore, miR-7-5p expression levels were examined in the human colon epithelial cell line HCEC 1CT and CRC cell lines HCT-116, SW480 and SW620. It was identified that the expression level of miR-7-5p was significantly lower in HCT-116, SW480 and SW620 cells compared with HCEC 1CT cells (Fig. 1B).

**Clinical significance of miR-7-5p expression in CRC.** The enrolled patients were classified into high or low miR-7-5p

expression groups based on the expression level of miR-7-5p. The 75th percentile of the  $2^{-\Delta\Delta C_t}$  values was used as the cut-off point (0.41) for patients with high or low miR-7-5p expression (21). Subsequently, associations between miR-7-5p expression and clinicopathological features were analyzed and it was revealed that low miR-7-5p expression was significantly associated with tumor size and tumor stage (22), however, it was not associated with age and sex (Table I). Furthermore, analysis of miR-7-5p expression on overall survival using Kaplan-Meier curve and log-rank test revealed that low miR-7-5p expression predicts a poor overall survival in patients with CRC compared with high expression (Fig. 1C).

**miR-7-5p inhibits CRC cell proliferation and migration *in vitro*.** The SW480 cell line exhibited the lowest miR-7-5p expression level among the CRC cell lines investigated, therefore, SW480 cells were selected for *in vitro* miR-7-5p biological function analysis. The miR-7-5p mimic, miR-7-5p inhibitor and NC-miR were used to regulate the expression of miR-7-5p in SW480 cells. It was confirmed

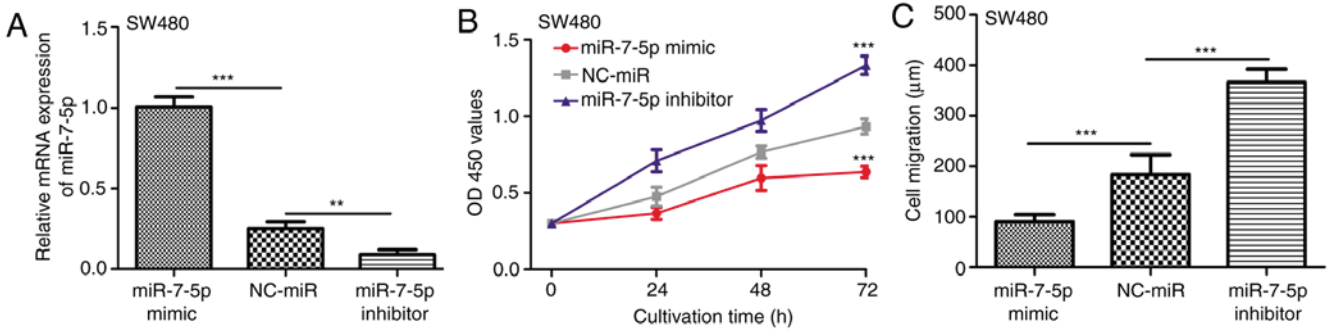


Figure 2. miR-7-5p inhibits cell proliferation and migration of SW480 cells. (A) miR-7-5p expression levels in SW480 cells following transfection with miRNAs were analyzed by reverse transcription-quantitative polymerase chain reaction. \*\**P*<0.01; \*\*\**P*<0.001. (B) Influence of miR-7-5p on SW-480 cell proliferation was analyzed by Cell Counting Kit-8 assay. \*\*\**P*<0.001 vs. NC-miR. (C) Influence of miR-7-5p on SW-480 cell migration was analyzed by a wound healing assay. \*\*\**P*<0.001. Data are presented as the mean ± standard deviation. miR-7-5p, microRNA-7-5p; miRNA, microRNA; NC, negative control; OD, optical density.

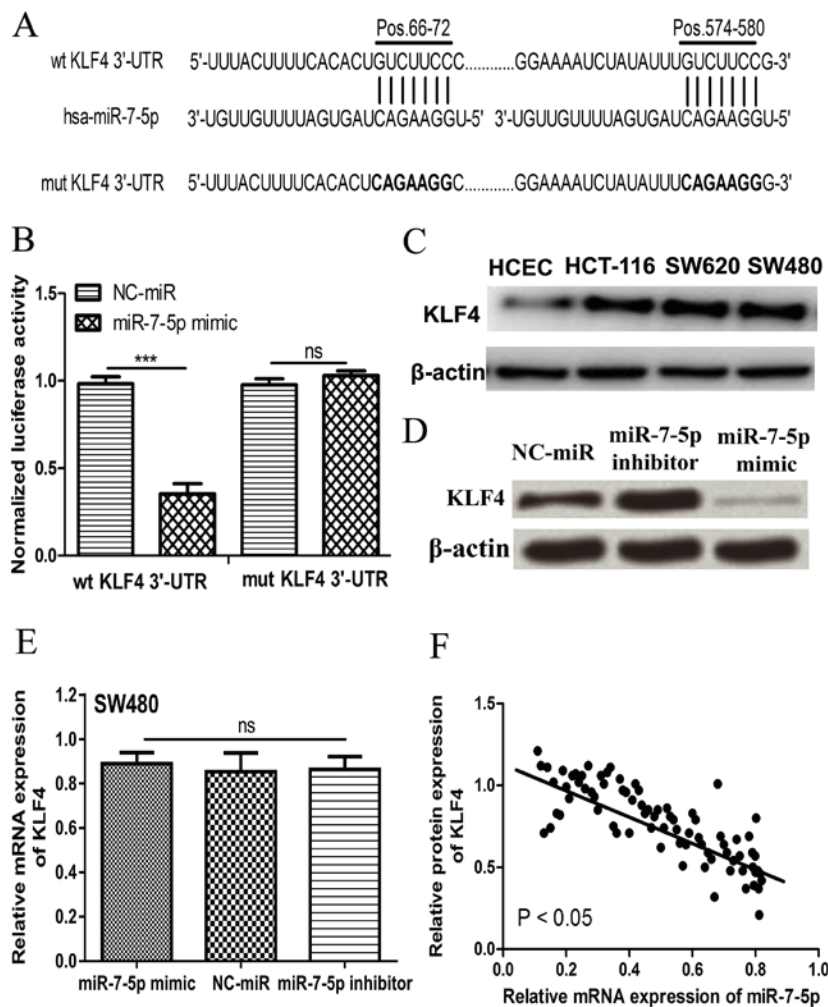


Figure 3. KLF4 is a direct target of miR-7-5p. (A) The predicted miR-7-5p binding sites on the 3'-UTR of KLF4 and the corresponding mutations. (B) Luciferase activities of wt and mut KLF4 3'-UTR reporter in SW-480 cells following transfection with miRNAs. (C) Western blot analysis of KLF4 expression in CRC cell lines (HCT-116, SW480 and SW620) and the HCEC ICT cell line. (D) Western blot analysis and (E) reverse transcription-quantitative polymerase chain reaction analysis of KLF4 expression in SW-480 cells following transfection with miRNAs. (F) Correlation analysis was performed to detect the association between miR-7-5p and KLF4 expression in CRC tissues. Data are presented as the mean ± standard deviation. \*\*\**P*<0.001. KLF4, Krüppel-like factor 4; miR-7-5p, microRNA-7-5p; miRNA, microRNA; wt, wild-type; mut, mutant; NC, negative control; NS, not significant.

that the expression level of miR-7-5p was enhanced by miR-7-5p mimic and reduced by miR-7-5p inhibitor (Fig. 2A). Subsequently, CCK-8 and wound healing assays revealed that SW480 cells transfected with miR-7-5p mimic

exhibited significantly lower levels of cell proliferation and migration compared with those transfected with NC-miR (Fig. 2B and C). Furthermore, downregulation of miR-7-5p in SW480 cells by miR-7-5p inhibitor increased the levels

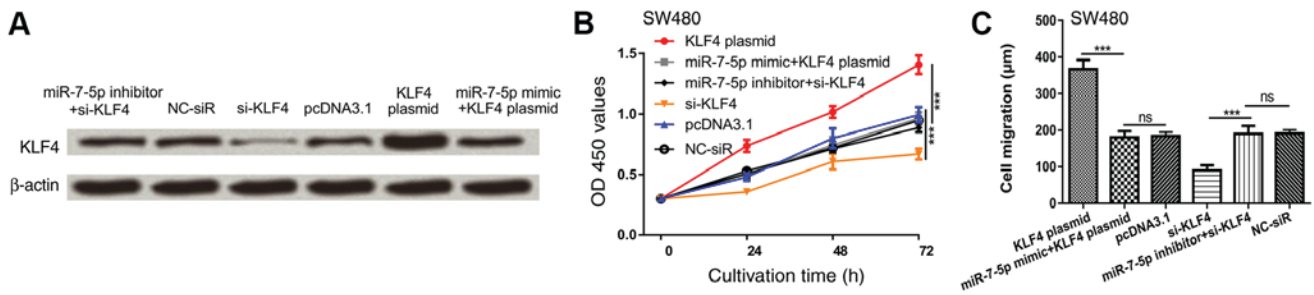


Figure 4. KLF4 is a mediator for the inhibitory effect of miR-7-5p on cell proliferation and migration. (A) Western blot analysis of KLF4 expression in SW-480 cells following transfection with siRNAs or KLF4 plasmid. (B) Influence of miR-7-5p and KLF4 on SW-480 cell proliferation was analyzed by Cell Counting Kit-8 assay. \*\*\* $P < 0.001$  KLF4 plasmid group vs. pcDNA3.1 or miR-7-5p mimic+KLF4 plasmid group; \*\*\* $P < 0.001$  si-KLF4 vs. NC-siR or miR-7-5p inhibitor+si-KLF4 group. (C) Influence of miR-7-5p and KLF4 on SW-480 cell migration was analyzed by wound healing assay. Data are presented as the mean  $\pm$  standard deviation. \*\*\* $P < 0.001$ . KLF4, Krüppel-like factor 4; miR-7-5p, microRNA-7-5p; si, small interfering; NC, negative control; OD, optical density; ns, not significant.

of proliferation and migration compared with the NC-miR group (Fig. 2B and C).

**miR-7-5p directly targets KLF4 in CRC.** Analysis using TargetScan demonstrated that KLF4, with two binding sites in its 3'-UTR, may be a target of miR-7-5p (Fig. 3A). Luciferase activity reporter assay was performed to confirm this prediction. It was revealed that miR-7-5p mimic significantly inhibited the luciferase activities of wt 3'-UTR of KLF4, however, it did not affect the luciferase activity of Mut 3'-UTR of KLF4 (Fig. 3B). Furthermore, it was identified that KLF4 expression was markedly higher in CRC cell lines compared with the HCEC 1CT cell line (Fig. 3C). Additionally, the expression level of KLF4 was decreased by miR-7-5p mimic but increased by miR-7-5p inhibitor in SW480 cells (Fig. 3D). However, miR-7-5p mimic and miR-7-5p inhibitor did not affect the mRNA expression level of KLF4, which suggests that miR-7-5p regulates KLF4 expression at the posttranscriptional phase (Fig. 3E). In addition, it was identified that KLF4 protein expression was inversely correlated with miR-7-5p expression in CRC tumor tissues (Fig. 3F).

**miR-7-5p inhibits CRC proliferation and migration partly by regulating KLF4.** To explore whether KLF4 serves as a mediator for the suppressive role of miR-7-5p, si-KLF4 and KLF4 plasmid were introduced into the SW480 cell line. As expected, KLF4 protein expression was enhanced by KLF4 plasmid but reduced by si-KLF4 (Fig. 4A). The inhibitory effects of miR-7-5p mimic on KLF4 expression could be reversed by KLF4 plasmid and the stimulatory effects of miR-7-5p inhibitor on KLF4 expression could be reversed by si-KLF4 (Fig. 4A). Cell proliferation and migration were enhanced following transfection with KLF4 plasmid but decreased following transfection with si-KLF4 (Fig. 4B and C). KLF4 overexpression significantly reversed the inhibition effects of miR-7-5p mimic on SW480 cell proliferation and migration (Fig. 4B and C). Furthermore, downregulation of KLF4 reversed the stimulation effects of miR-7-5p inhibitor on SW480 cell proliferation and migration (Fig. 4B and C). These results indicated that miR-7-5p inhibits CRC proliferation and migration in part via regulating KLF4.

## Discussion

KLF4 is a zinc-finger transcription factor that functions as either a tumor suppressor or an oncogene in human cancer (23). Previous studies have identified that KLF4 serves a critical role in regulating malignant cell behaviors by activating or repressing the expression of downstream target genes (24-27). Furthermore, accumulating evidence has suggested that KLF4 functions as an oncogene in CRC (18,19). However, to the best of knowledge, the molecules that regulate KLF4 expression remain to be investigated.

A number of studies have demonstrated that miR-7-5p expression is frequently downregulated in several types of tumor (12-14). The present study demonstrated that miR-7-5p expression was downregulated in CRC cell lines and tissues, which, to the best of our knowledge, is the first study to reveal the expression pattern of miR-7-5p in CRC. Furthermore, the results demonstrated that patients with low miR-7-5p expression exhibited a larger tumor size, advanced tumor stage and worse 5-year overall survival. These results suggest that low miR-7-5p expression is associated with aggressive tumor behaviors.

Identifying the targets of miRs in tumors is crucial for understanding the importance of miRs in tumor progression and may provide promising antitumor therapeutic targets (18,19). KLF4, whose expression is upregulated in CRC (18,19), was predicted as a potential target of miR-7-5p. A luciferase activity assay validated KLF4 as a direct target of miR-7-5p. Notably, it was identified that KLF4 expression was inversely correlated with miR-7-5p in CRC tumor tissues. It should be noted that abnormal status of cell proliferation and cell migration are two major characteristics of malignant cells (8). Therefore, the current study investigated the effects of miR-7-5p on CRC cell proliferation and cell migration *in vitro*. miR-7-5p overexpression significantly inhibited cell proliferation and migration. Furthermore, it was revealed that overexpression or downregulation of KLF4 could partially restore the effects of miR-7-5p mimic or inhibitor on cell behaviors. These findings demonstrated that miR-7-5p regulates CRC cell proliferation and migration by targeting KLF4.

In conclusion, the present study provided a novel insight into the mechanisms underlying human CRC progression.

Decreased miR-7-5p expression was positively associated with CRC progression by directly targeting the expression of KLF4. Identification of this association may provide a novel therapeutic approach for CRC.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

YDX conceived and deigned the whole study. MJD and YMX performed the study and were major contributors in writing the manuscript. YDX reviewed the manuscript. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

This study was approved by the Ethics Committee of The No. 2 Hospital of Ningbo (Ningbo, China). Written informed consent was obtained from all enrolled patients.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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