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Original Article

MicroRNA-486-5p targets FGF9 and inhibits colorectal cancer proliferation, migration and invasion

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Abstract: Colorectal cancer (CRC) is one of the most common malignancies worldwide, and microRNAs (miRNAs), which act as tumor suppressors or oncogenes, are involved in CRC development and progression. The purpose of this study was to investigate the function and potential mechanism of miR-486-5p in CRC. The results showed that miR-486-5p was significantly down-regulated in CRC cell lines and tissues. Over-expression of miR-486-5p significantly inhibited cell proliferation, migration and invasion *in vitro* and suppressed tumor growth *in vivo*. Fibroblast growth factor 9 (FGF9) was identified as a direct target of miR-486-5p in CRC cells, and FGF9 expression was negatively correlated with miR-486-5p expression in clinical specimens. Enforced expression of FGF9 significantly reversed the tumor suppressive effects of miR-486-5p. Taken together, these findings revealed a functional and mechanistic link between miR-486-5p and FGF9 in the pathogenesis of CRC and miR-486-5p has potential as a therapeutic target for CRC.

Keywords: Colorectal cancer, miR-486-5p, FGF9, proliferation, invasion

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide with a high incidence and mortality rate [1, 2]. Despite advances in therapeutic strategies, the clinical outcome and prognosis of CRC remains poor [3]. Tumor metastasis is a major cause of mortality in the multi-step genetic events involved in CRC [4, 5]. Although many cell growth and metastasis-related genes, including p53, APC and K-ras, have been identified in CRC, the molecular mechanisms that suppress tumor cell growth, migration and invasion are largely unknown. Recent studies have revealed that the non-coding microRNAs (miRNAs) are novel regulators of tumor progression and novel targets for therapy in CRC [6].

miRNAs are endogenous non-coding RNAs containing ~22 nucleotides (nt) that can negatively regulate protein expression by inducing the degradation of target mRNAs or impairing their translation or both by specifically binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs [7, 8]. miRNAs participate in multiple cancer cell biological processes, such as proliferation, cell cycle, invasion and apoptosis [9, 10]. Accumulating data point to a central regulatory role for miRNAs in the initiation and development of CRC, and these miRNAs may function as tumor suppressors or oncogenes [11, 12]. Many miRNAs have been proved to play important roles in human CRC, including miR-224, miR-320a, miR-135, miR-154 and miR-200 [4, 6, 13-15].

miR-486-5p is a recently discovered miRNA and is involved in tumorigenesis. miR-486-5p was reported to be down-regulated in non-small cell lung cancer [16], gastric adenocarcinoma [17], lung cancer [18], breast cancer [19] and hepatocellular carcinoma [20], and acts as a tumor suppressor. In contrast, it has also been shown to be up-regulated in renal cell carcinoma [21] and functions as an oncogene. However, the role of miR-486-5p in CRC tumorigenesis remains undefined.

In this study, we aimed to determine the potential regulatory mechanisms of miR-486-5p in CRC. We showed that miR-486-5p is down-regulated in CRC, and its over-expression inhibited proliferation, migration and invasion *in vitro* and

suppressed tumor growth *in vivo* by binding to FGF9 3' UTR in CRC cells. Our findings showed that the miR-486-5p/FGF9 axis is an important regulator in the development and progression of CRC and may be a candidate target for CRC treatment.

Materials and methods

Cell culture and preparation of clinical samples

Four human CRC cell lines (SW480, HCT116, SW620 and HT29) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen) at 5% CO_2 humidity and 37°C. The normal colon epithelial cell line FHC was grown in DMEM: F12 supplemented with 10% FBS (Invitrogen). Twenty CRC tissue samples and matched adjacent normal tissues were obtained from Jinshan Hospital affiliated to Fudan University, and frozen in liquid nitrogen and stored at -80°C until use. This study was approved by the Institutional Ethical Review Boards of our institute, and written informed consent was obtained from all patients.

RNA preparation and quantitative RT-PCR analysis

Total RNA was extracted from the cell lines and tissues using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA was reverse transcribed to cDNA using the PrimeScript® 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Quantitative PCR (qRT-PCR) was performed to determine the expression levels of miR-486-5p and FGF9 using SYBR Premix ExTaq (TaKaRa). U6 or β-actin was used as the control for normalization. The relative expression levels of the gene of interest were calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate.

Western blot analysis

Cells were lysed in RIPA buffer (Beyotime, Jiangsu, China) and the protein concentration was determined using the Pierce® BCA Protein Assay Kit (Thermo Fisher, CA, USA). Total protein was prepared and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto polyvinylidene difluoride membranes and blocked with 5% nonfat milk. The target proteins were detected according to standard methods using the following primary antibodies: anti-FGF9 (Santa Cruz, CA, USA), goat anti-peroxidase-conjugated secondary antibody (Sigma, St Louis, MO, USA). An anti-β-actin antibody (Sigma) was used as a loading control.

Oligonucleotide and plasmid transfection

miR-486-5p mimic and negative control (miR-NC) were designed and synthesized by RiboBio (Guangzhou, China). Cells were plated in individual wells of 6-well plates. miR-486-5p mimic or miR-NC was transfected into SW620 and HCT116 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h, the cells were harvested for the assays described below. The coding region of FGF9 mRNA was amplified by PCR from human genomic DNA and inserted into the multiple cloning site of expression vector pENTER (Invitrogen) and verified by sequencing. SW620 and HCT116 cells were transfected with the pEnter-FGF9 plasmid (FGF9) or empty pEnter-vector using Lipofectamine™ 2000. The cells were harvested 48 h after transfection for the specified assays. The FGF9 3' UTR was generated by PCR amplification and subcloned into the pGL3-basic luciferase reporter plasmid (Promega).

Cell proliferation assay

Cell viability was detected using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) assay. Cells were seeded on 96-well plates at a density of 3×10^3 cells per well. The viability of cells was determined for five consecutive days (day 1, 2, 3, 4, and 5) and measured at 450 nm using a microplate reader (Epoch; BioTek, Winooski, VT, USA). All experiments were repeated three times and the averages were calculated.

Cell migration and invasion assays

A Transwell assay (24-well plates, 8-mm pore size; Corning, Inc., Corning, NY, USA) coated with or without Matrigel (BD Biosciences, San Jose, CA, USA), respectively, on the upper surface of the membrane was used to determine the effect of miR-486-5p on migration and

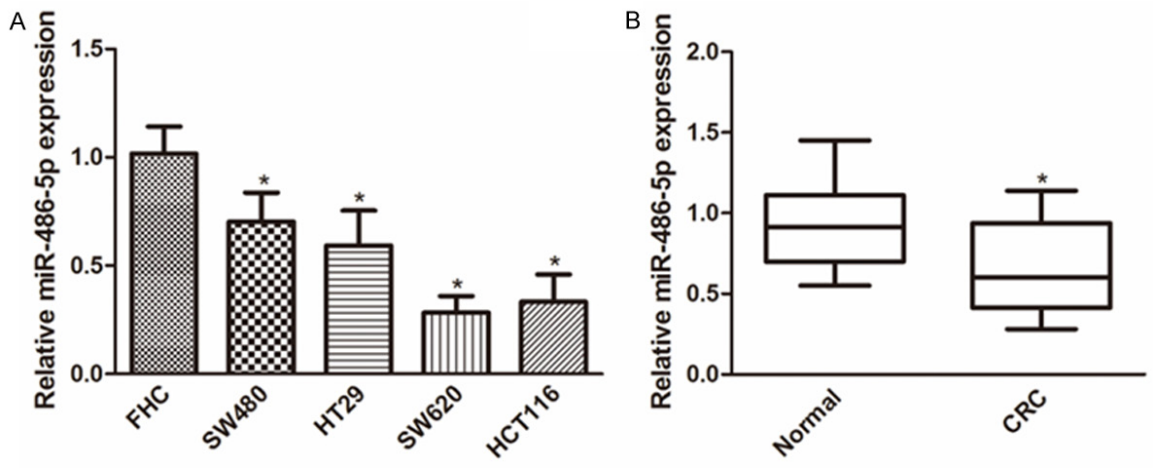

Figure 1. miR-486-5p is down-regulated in CRC cell lines and tissues. A. Expression of miR-486-5p in CRC cell lines and the normal colon epithelial cell line FHC was determined by qRT-PCR. B. qRT-PCR analysis showing miR-486-5p expression in CRC tissues and matched adjacent normal tissues.

invasion *in vitro* according to the manufacturer's protocol. 1×10^5 cells were placed in the top chamber in triplicate. For the invasion assay, the upper chamber was coated with Matrigel. The cells on the upper surface of the membrane were removed following incubation for 24 h, and the cells on the undersurface were fixed, stained with 0.1% crystalviolet and counted under a light microscope. The Transwell migration assay was performed in the same way as the invasion assay, but without the Matrigel coating.

Luciferase reporter assay

Cells were seeded in 24-well plates (1×10^5/well) in triplicate and cultured for 24 h. The pGL3-luciferase reporter gene plasmid pGL3-FGF9-3'-UTR (wild-type, WT) or the control-luciferase plasmid (Mutant) were co-transfected into the cells with the control pRL-TK Renilla plasmid (Promega, Madison, WI, USA) using Lipofectamine 2000 Reagent (Invitrogen). Luciferase and Renilla activities were assayed 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega) following the manufacturer's instructions [4].

Tumorigenicity in vivo

Male BALB/c nude mice aged 4-6 weeks were obtained from Shanghai Laboratory Animal Center of China. For the tumor growth assay, SW620 cells transfected with miR-486-5p mimic and miR-NC, were subcutaneously injected into nude mice. Tumor growth was determined by measuring the tumor volume, V= (mm3, V = tumor length × tumor width^2/2) every 3 days using calipers [22]. The animal studies and the experimental protocol were approved by the Institutional Animal Care and Use Committee of Fudan University. All animal experiments were performed according to the guidelines on the care and use of animals for scientific use.

Statistical analysis

All data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. The mRNA or protein relationship between miR-486-5p and FGF9 was analyzed by Pearson's correlation. The two-tailed Student's *t*-test was used to determine the *P*-value, and $P<0.05$ was considered significant.

Results

miR-486-5p was reduced in CRC cell lines and tissues

To understand the role of miR-486-5p in CRC, we determined the expression of miR-486-5p in CRC cell lines and tissues using qRT-PCR. As shown in **Figure 1A**, miR-486-5p expression was lower in CRC cell lines than in normal colorectal epithelial cells. miR-486-5p expression was also decreased in CRC samples compared with matched adjacent non-tumor tis-

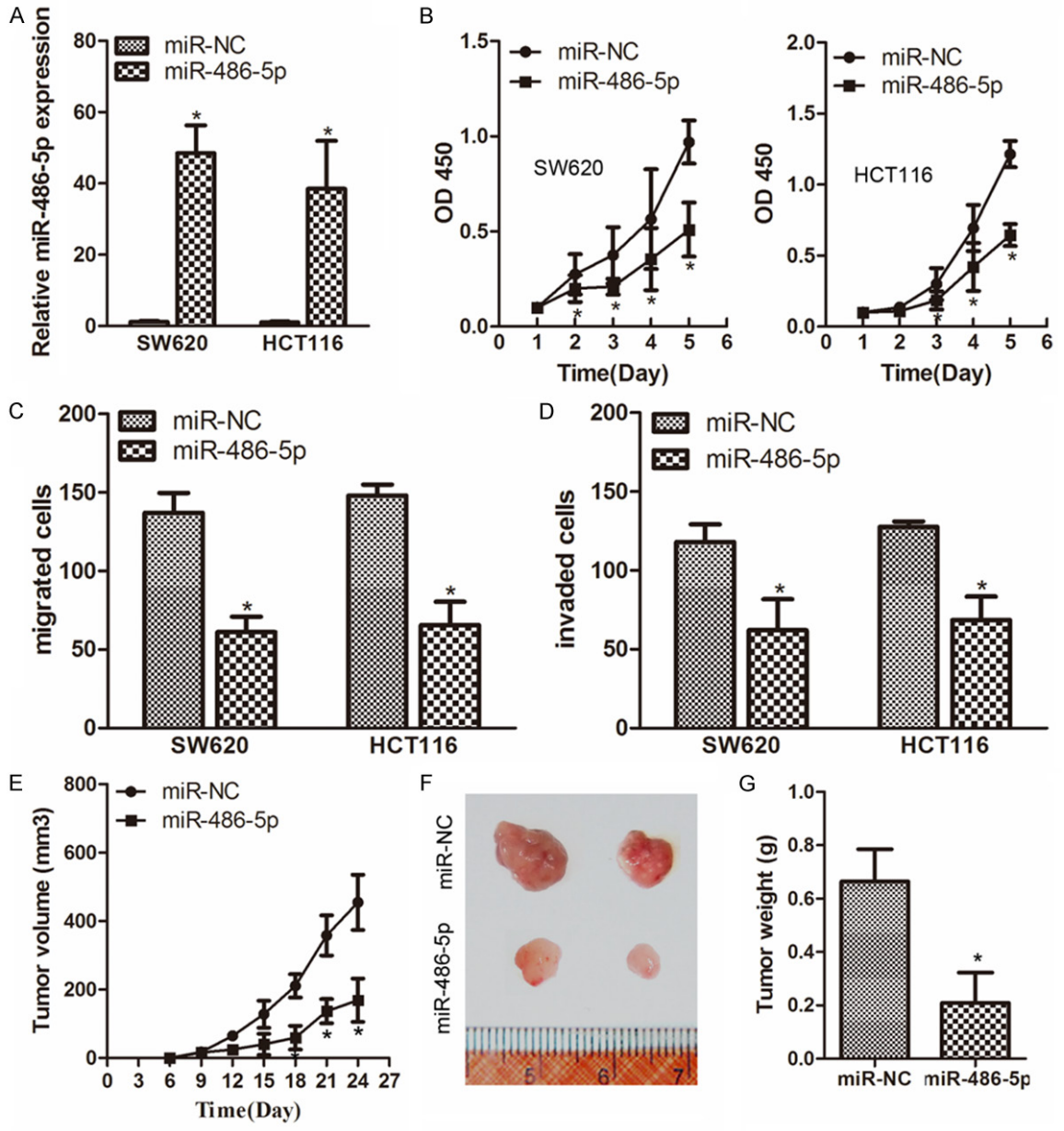

Figure 2. miR-486-5p inhibited cell proliferation, migration and invasion and suppressed tumorigenicity. (A) Relative expression of miR-486-5p was examined in SW620 and HCT116 cells transfected with miR-486-5p mimic or miR-NC by qRT-PCR. (B) Cell proliferation was examined by the CCK-8 assay. (C) Cell migration and (D) cell invasion were measured using Transwell assays. (E) Tumor volumes were measured on the indicated days. (F) Representative photograph of the formed tumors. (G) Tumor weight was calculated at 24 days post injection.

sues (**Figure 1B**). These data suggest that miR-486-5p was down-regulated in both CRC cell lines and tissues.

Ectopic expression of miR-486-5p inhibited cell proliferation, migration and invasion and suppressed tumorigenicity

To investigate the biological function of miR-486-5p in CRC, over-expression of miR-486-5p was achieved by transfecting miR-486-5p mimic into SW620 and HCT116 cells (**Figure 2A**). The CCK-8 assay indicated that over-expression of miR-486-5p significantly decreased the growth rate of SW620 and HCT116 cells compared with the negative control (**Figure 2B**). Transwell migration and Matrigel invasion assays were used to evaluate the effects of miR-486-5p on cell migration and invasion. The results showed that cell migration

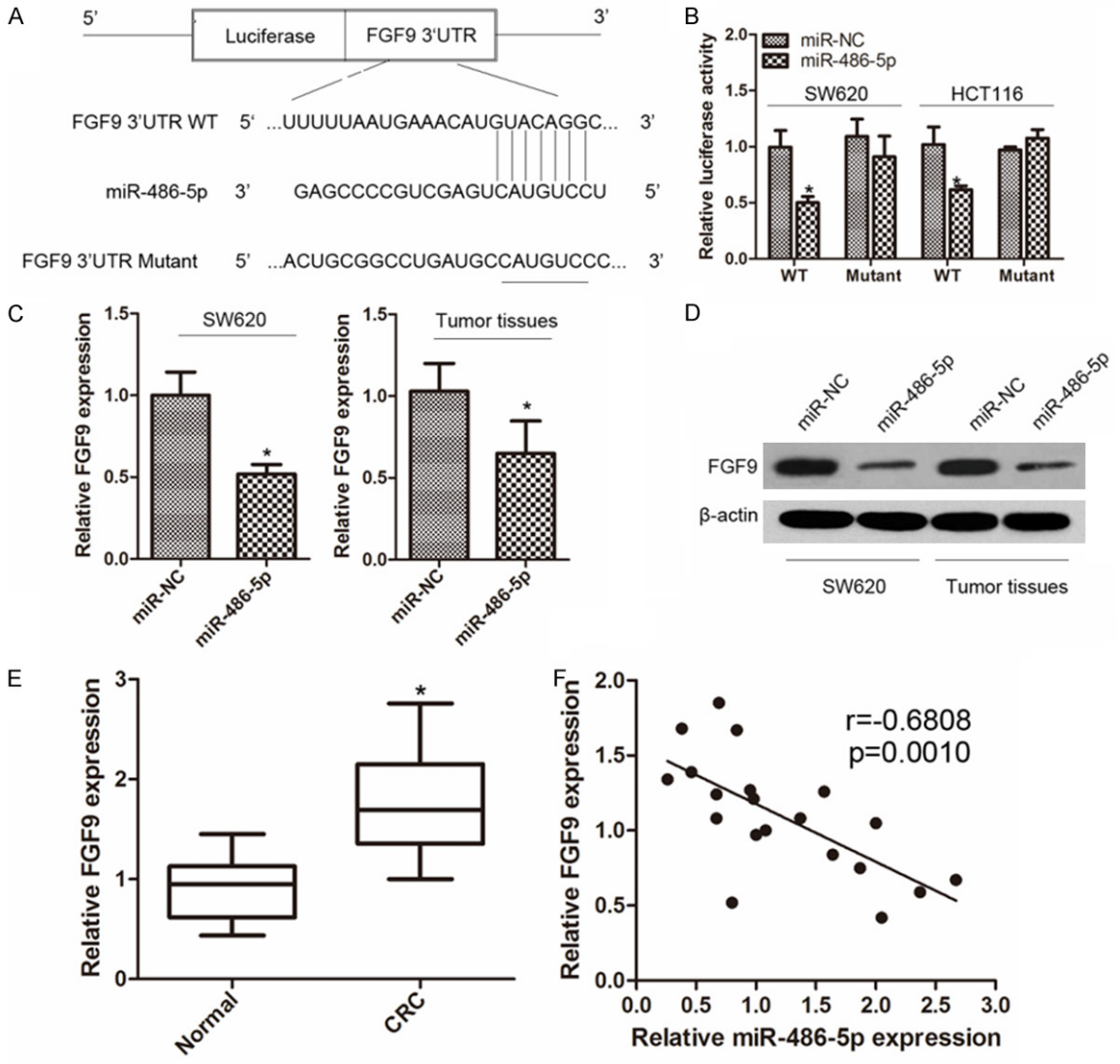

Figure 3. FGF9 is a target of miR-486-5p in CRC cells. A. miR-486-5p binding sites and mutation of the 3'-UTR of FGF9 mRNA. B. Functional identification of miR-486-5p binding sites in the 3'-UTR of FGF9 mRNA. Luciferase activity was used as a reporter for determination of the miR-486-5p binding site in the 3'-UTR. WT or Mutant FGF9 3'-UTR reporter genes were transfected into SW620 and HCT116 cells in combination with miR-486-5p mimic or miR-NC and the luciferase activity was assessed. C, D. The expression of FGF9 in SW620 cells and tumor tissues transfected with miR-486-5p mimic or miR-NC was examined by qRT-PCR and western blotting. E. Expression of FGF9 in CRC tissues and paired non-tumor tissues. F. Correlation between miR-486-5p and FGF9 mRNA expression in CRC tissues. (Spearman's correlation analysis, r = -0.6808, P = 0.0010).

and invasion were inhibited in the miR-486-5p mimic group compared with the negative control group, thus demonstrating the inhibitory effects of miR-486-5p on CRC cells (**Figure 2C**, **2D**).

We next examined the effects of miR-486-5p on the tumorigenicity of CRC cells. SW620 cells transfected with miR-486-5p mimic or miR-NC were subcutaneously injected into the flanks of nude mice, respectively. The tumors were measured until the mice were killed on day 24. Over-expression of miR-486-5p suppressed tumor growth compared with the negative control (**Figure 2E**) and tumor size in the miR-486-5p mimic group was significantly smaller than that in the miR-NC group (**Figure 2F**). Similar results were observed for tumor weight (**Figure 2G**). These findings indicate that miR-486-5p functions as a tumor suppressor in CRC.

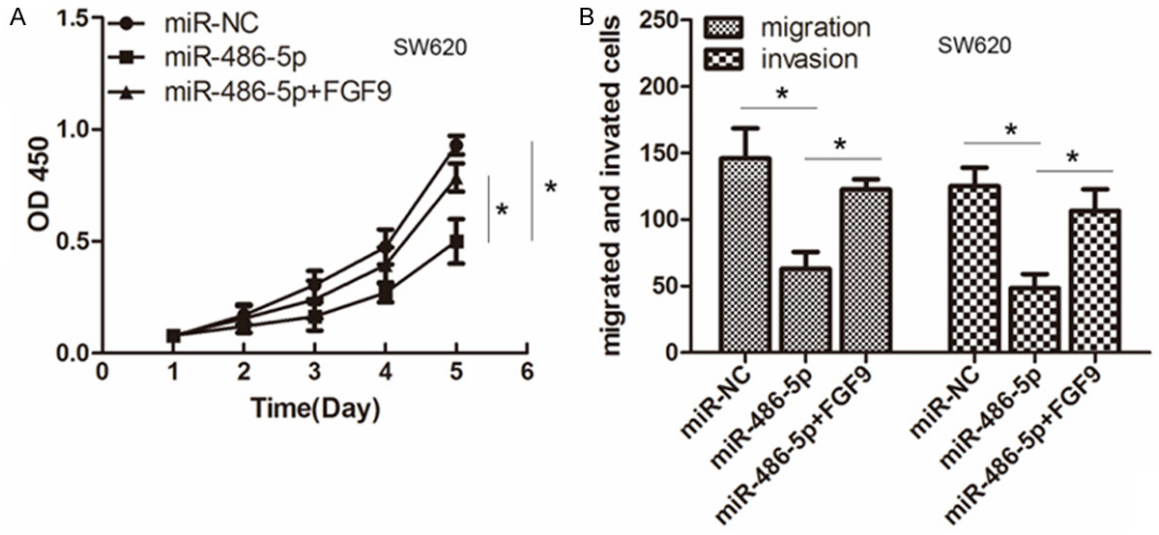

Figure 4. FGF9 attenuated the suppressive effect of miR-486-5p. Exogenous expression of FGF9 (encoding FGF9 without the 3'UTR) and miR-486-5p co-transfected into SW620 and HCT116 cells. A. The CCK-8 assay was used to examine proliferation. B. Migration and invasion ability was determined by Transwell assays.

miR-486-5p suppressed the expression of FGF9 by targeting its 3'-UTR

To elucidate the underlying molecular mechanisms by which miR-486-5p executes its function, we next identified the target genes of miR-486-5p using the publicly available databases (TargetScan and miRanda). FGF9 was predicted to be a target of miR-486-5p and one putative binding site in the FGF9 3'-UTR is shown in **Figure 3A**. The luciferase reporter assay was then used to determine whether miR-486-5p directly binds to the 3'-UTR of FGF9. As demonstrated in **Figure 3B**, compared with miR-NC, miR-486-5p significantly suppressed the luciferase activity of the WT3'-UTR, while mutation of the miR-486-5p binding sites blocked this inhibition. In addition, we analyzed the level of FGF9 expression in SW620 cells transfected with miR-486-5p mimic or miR-NC and in tumors isolated from nude mice using qRT-PCR and Western blotting. The results showed that miR-486-5p significantly inhibited the expression of FGF9 levels (**Figure 3C**, **3D**). Next, we determined FGF9 expression in CRC tissues and adjacent normal tissues. qRT-PCR analysis indicated that FGF9 expression was significantly increased in CRC tissues compared with the paired normal tissues (**Figure 3E**). Statistical analysis showed that the level of miR-486-5p was inversely correlated with the level of FGF9 in human CRC tissues (**Figure 3F**). These results confirmed that FGF9 is a target of miR-486-5p.

Enforced expression of FGF9 alleviates the effects of miR-486-5p on proliferation, migration and invasion

Given that FGF9 is a direct target of miR-486-5p, we investigated whether over-expression of FGF9 reversed the suppressive effect of miR-486-5p. The FGF9 expressing vector (which encodes the entire coding region of FGF9 without 3' UTR) was transiently introduced in SW620 cells co-transfected with miR-486-5p. The results showed that SW620 cells transfected with FGF9 and miR-486-5p grew faster (**Figure 4A**) and had stronger migration and invasion (**Figure 4B**) ability than those transfected with empty vector and miR-486-5p. These results indicated that over-expression of FGF9 significantly alleviated the suppressive effects of miR-486-5p on CRC cells.

Discussion

It is now widely accepted that miRNAs contribute to cancer development by acting as oncogenes or tumor suppressor genes. Previous studies have shown that miR-486-5p is dysregulated in several cancers and its potential function has also been partly evaluated in several studies [18-21, 23]. For example, Wang *et al.* reported that down-regulation of miR-486-5p

contributes to tumor progression and metastasis by targeting pro-tumorigenic ARHGAP5 in lung cancer [18]. Zhang *et al.* found that miR-486-5p expression was significantly down-regulated in breast cancer tissues and cell lines, and over-expression of miR-486-5p markedly suppressed breast cancer cell proliferation *in vitro* and *in vivo*, induced G0/G1 arrest, and promoted apoptosis [19]. Huang *et al.* revealed that miR-486-5p suppressed hepatocellular carcinoma cell proliferation, migration and invasion *in vitro* and inhibited hepatocellular carcinoma growth *in vivo* [20]. In contrast, Goto *et al.* showed that miR-486-5p was up-regulated in renal cell carcinoma [21]. A previous study reported that miR-486-5p was down-regulated in CRC samples compared with matched non-tumor tissues [23]. However, the function of miR-486-5p in CRC pathogenesis, as well as the molecular mechanisms by which miR-486-5p exerts its function and modulates the malignant phenotypes of CRC cells, is not fully understood.

In the present study, miR-486-5p expression was decreased in CRC cell lines and tissues compared with the negative controls. Ectopic expression of miR-486-5p suppressed cell proliferation, migration and invasion of CRC cells *in vitro* and inhibited tumorigenesis *in vivo*. These findings revealed that miR-486-5p is a potential suppressor of CRC and further work was carried out to characterize the mechanism. TargetScan and miRanda were used to identify potential gene targets of miR-486-5p and revealed that FGF9 was a potential target of miR-486-5p in CRC.

The fibroblast growth factor (FGF) family includes at least 24 distinct polypeptides with molecular masses ranging from 17 to 34 kDa and share 13-71% sequence identity [24]. Many mammalian FGFs are abundantly expressed in a specific spatial and temporal pattern and are involved in many cellular processes, including development [25] and angiogenesis [26]. FGF9, a secretory protein of the FGF family, is reportedly expressed in stromal cells [27-29]. Studies have also demonstrated that FGF9 exhibits mitogenic activity in glioma [30], and in epithelial and fibroblast cells [31]. Over-expression of FGF9 has transforming potential in NIH3T3 fibroblasts and stimulates the invasion of epithelial and endothelial cells [32]. This suggests that FGF9 over-expression may result in uncontrolled cell proliferation and malignancy. FGF9 has been implicated in various cancers such as ovarian endometrial adenocarcinoma [31], hepatocellular carcinoma [33], prostate carcinoma [34] and gastric cancer [35]. A previous study reported that the expression of FGF9 was strong in a subset of advanced colon cancers, and over-expression was negatively correlated with patient survival [36]. In the present study, we demonstrated that FGF9 was a direct target of miR-486-5p in CRC cells using luciferase reporter assays. FGF9 expression was negatively correlated with miR-486-5p expression in CRC tissues. Furthermore, over-expression of FGF9 alleviated the suppressive effects of miR-486-5p. These results suggest that miR-486-5p inhibited CRC growth and metastasis partly by repressing FGF9 expression.

In summary, the current study revealed that miR-486-5p is down-regulated in CRC tissues and cell lines. We provide evidence that miR-486-5p is involved in CRC proliferation, migration and invasion *in vitro* and suppressed tumor growth *in vivo*, potentially *via* the direct modulation of the downstream target FGF9. These results indicate that miR-486-5p plays an important role during CRC carcinogenesis and may serve as a putative target for CRC diagnosis and therapy.

Disclosure of conflict of interest

None.

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