MicroRNA-7 suppresses human colon cancer invasion and proliferation by targeting the expression of focal adhesion kinase

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Abstract. Previous studies have demonstrated that microRNA (miRNA) are essential in tumor development and invasion. The close association between focal adhesion kinase (FAK) and colon cancer (CC) has been previously reported. miRNA-7 (miR-7) inhibits the translation of FAK protein. Therefore, the present study aimed to assess the underlying molecular mechanism of miR-7 in human CC cell lines, to provide a novel therapeutic biomarker of CC in the future. The present study detected the expression of miR-7 in 60 CC tissues by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The association between the expression of miR-7 and clinical pathological factors was analyzed. Overexpression/underexpression of miR-7 were established by transfecting miR-7mimics/inhibitors into HCT-8 and Caco-2 cells. The transfected CC cell lines were used in cell viability and scratching assays. The regulation of FAK by miR-7 was analyzed by western blotting and RT-qPCR. It was demonstrated that the expression of miR-7 negatively correlated with lymph node metastasis and tumor node metastasis staging in CC ($P<0.05$). Inhibition of miR-7 led to an accelerated ability of proliferation and migration in CC cell lines. Additionally, overexpression of miR-7 inhibited the proliferation and migration of CC cells. In addition, it was also observed that miR-7 regulated the proliferation and migration of CC by regulating the protein expression of FAK, therefore, regulating the expression of matrix metalloproteinase (MMP)-2 and MMP-9. miR-7 inhibited the proliferation and migration of CC cells by regulating FAK. These findings suggested that miR-7 may be a novel therapeutic target for CC.

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Introduction

Colon cancer (CC) is the second leading cause of cancer-associated mortality worldwide (1). Invasion is the most difficult problem when treating CC, however, the molecular mechanism underlying CC invasion remains to be fully elucidated. According to previous data, 10-25% of patients with CC have been diagnosed with liver metastasis (2-4). The treatments for CC include surgery, chemotherapy, endoscopic stent implant and targeted chemotherapy (5,6). The most difficult problems for the patients with CC are invasion and metastasis. Early detection and targeted therapy are more practical and important for the treatment of CC.

Focal adhesion kinase (FAK) is predominantly distributed in the cell cytoplasm, and was initially identified in the v-src transfected chicken embryo fibroblast. FAK causes the accumulation and depolymerization of cytoskeleton proteins, therefore, influencing the structures of cell adhesion sites and membrane protrusions to regulate cell activities (7). FAK also promotes the secretion of the matrix metalloproteinases (MMPs), which can damage the extracellular matrix components of the intercellular matrix and basement membrane, therefore, accelerating the growth and invasion of CC cells (8). FAK is involved in breast cancer, CC, prostate cancer and other malignant tumor types (9,10), and is markedly associated with tumor proliferation, apoptosis, adhesion and migration.

MicroRNA (miRNA), a type of highly conserved non-coding RNA, consists of 18-25 nucleotides with endogenous single-stranded RNA. Lee *et al* (11) identified the first miRNA, *let4*, in *Caenorhabditis elegans* in 1993. miRNA (miR)-21 and miR-264 were subsequently identified in vertebrates, flies, worms, viruses and certain plant species. It is estimated that there are >1,000 miRNAs in the human genome (12). miRNAs have been demonstrated to be closely associated with tumor growth and metastasis.

Therefore, miRNAs may be targets for cancer diagnosis and treatment (13). miR-7 is important for the growth and metastasis of glioma, breast, ovarian, bladder, gastric, lung, liver and colon cancer (14-20). miR-7 inhibits the invasion of glioma cells by binding to the 3-untranslated region of FAK mRNA, which inhibits the protein translation of FAK, leading to decreased invasion of glioma cells (14).

The present study investigated the expression of miR-7 in CC tissues and adjacent colon tissues. Additionally, the possible

correlations of miR-7 and the clinical pathological factors in CC were determined, illustrating the effects of overexpression or underexpression of miR-7 on the growth and migration of CC *in vitro*. These findings may provide novel targets for the treatment of CC.

Materials and methods

Antibodies and reagents. The miR-7 mimic (miR-7m), negative-5'-cy3, miR-7 inhibitor (miR-7i) and the negative control (NC) were purchased from Ribobio Co., Ltd. (Guangzhou, Guangdong, China). Antibodies were purchased as follows: Rabbit monoclonal FAK (1:1,000; Abcam, Cambridge, MA, USA; cat. no. ab40794), rabbit polyclonal anti-MMP-2 (1:1,000; Proteintech Group, Inc., Chicago, IL, USA; cat. no. 10373-2-AP) and rabbit polyclonal anti-MMP-9 (1:1,000; Proteintech Group, Inc.; cat. no. 10375-2-AP), and rabbit polyclonal anti-actin (1:5,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-1616). All antibodies were diluted with phosphate-buffered saline (PBS). Enhanced chemiluminescence western blot detection reagents were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Protease inhibitor cocktail, Lubrol-PX and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). An Invitrogen cDNA synthesis kit was purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). All RNA duplexes were chemically synthesized by GenePharma Co., Ltd. (Shanghai, China).

Cell culture and tissue specimens. Human HCT-8 and Caco-2 CC cell lines were obtained from American Type Culture Collection (Rockville, IL, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco Life Technologies) and 100 μg/ml streptomycin (NCPC Hebei Huamin Pharmaceutical Co., Ltd., Hebei, China) in a humid incubator with 5% CO_2 at 37˚C.

Paired CC samples and adjacent normal colon tissues were obtained from 60 patients who were diagnosed with CC between 2011 and 2012 in the Department of Pathology, The First Affiliated Hospital of Nanchang University (Nanchang, China). No patient received chemotherapy or radiotherapy prior to surgery and patients were diagnosed by clinical pathological staging based on the 2010 American Joint Committee on Cancer criteria (21). All patients provided written informed consent prior to surgery. The detailed clinical pathological information of all patients and their correlation with the expression of miR-7 are summarized in the Table I. The present study was approved by the institutional review board of The First Affiliated Hospital of Nanchang University medical research ethics committee.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was isolated using Invitrogen TRIzol reagent (Thermo Fisher Scientific). The RNA was subsequently reverse transcribed into cDNA using the stem-loop reverse transcription primer (Ribobio Co., Ltd.) for miRNA detection. RT was performed using Revert Aid™ reverse transcriptase (Fermentas, Ontario, Canada) according to the manufacturer's instructions. The cDNA results were quantified by SYBR Premix Ex Taq™ (Takara Bio., Inc., Otsu, Japan). The mRNA expression levels of miR-7 and FAK were detected by RT-qPCR and analyzed on an Applied Biosystems 7500 PCR Detection system (Applied Biosystems, Inc. Foster City, CA, USA). The mRNA expression levels of miR-7 and FAK were determined and normalized against the expression of U6 using the comparative cycle threshold (Ct) method (22).

Transient transfection of miR-7m or miR-7i. The human HCT-8 and Caco-2 CC cell lines were cultured in antibiotic-free medium for 48 h in 6-well plates. Upon reaching a confluence of 60-70%, the miR-7m (50 nM), miR-7i (300 mM), unrelated sequence positive control (50 nM) or the NC (300 nM) were transfected into the cells using an Invitrogen Lipofectamine 2000 reagent (Thermo Fisher Scientific), according to the manufacturer's instructions.

Protein extraction and western blotting. Following incubation for 72 h after transfection with miR-7m, miR-7i or the control, the cells were washed with cold PBS three times and were subsequently resuspended in protein lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) at 4˚C for 30 min. The protein concentrations were measured following centrifugation and quantified using the bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). A Bio-Rad Model 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure the results of the assay. An equal quantity of protein was separated on 8% SDS-PAGE gels (Sigma-Aldrich) and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 50 mg/ml non-fat milk in 10 ml PBS for 2 h at room temperature and were subsequently incubated overnight at 4˚C with the appropriate primary antibody. The membranes were washed with PBS and incubated with horseradish peroxidase-conjugated goat anti-rat immunoglobulin (Ig)G/IgM (cat. no. AP132P; 1:1,000; Merck Millipore, Darmstadt, Germany) at 4˚C for 4 h. The images were captured and quantified using Gel Doc XR-Z system (controlled by Image Lab™ version 2.0; (Bio-Rad Laboratories, Inc.).

Cell viability analysis. A cell viability assay was performed on the CC cells using an MTT reduction assay (23). The cells were seeded into 96-well plates for 24 h and were subsequently transfected with miR-7m/i. At 24, 48 and 72 h after transfection, 20 μl MTT reagent (5 mg/ml) was added to each well for 4 h at 4˚C. The generated formazan product was dissolved in 150 μl dimethyl sulfoxide. The optical density at 490 nm was measured using the Bio-Rad Model 680 microplate reader. Triplicate wells were used for each cell line and experiments were repeated in triplicate.

Wound healing assay. Cell migration was measured using a wound healing assay. The HCT-8 and Caco-2 cells ($3x10^5$/well) were seeded into 6-well plates. Once the cells reached a confluence between 90 and 100% at 48-72 h after transfection, a 10-μl pipette tip was used to scrape the cell monolayer. Fresh serum-free medium was added and images

Table I. Correlation between the expression of miR-7 and clinical pathological characteristics in colon cancer.

Characteristic	Cases (n)	miR-7 expression[b]	Spearman's (R)	P-value
Gender				
Male	29	0.38 (0.11-2.27)	-0.726	0.773
Female	31	0.58 (014-2.01)		
Age (years)				
<60	28	0.44 (0.15-2.56)	-1.089	0.276
≥60	32	0.49 (0.10-1.71)		
Histopathologic differentiation				
WD/MD	36	0.35 (0.12-1.97)	-0.800	0.424
PD/UD	24	0.49 (0.13-1.29)		
Lymph node metastasis				
Yes	27	0.19 (0.09-1.16)	-2.290	0.022[a]
No	33	0.69 (0.27-2.23)		
TNM staging				
I+II	30	0.75 (0.30-2.32)	-2.698	0.007
III+IV	30	0.17 (0.09-1.23)		

[a]Significant difference; [b]A quarter to three quarter enclosed in parentheses. WD/MD, well differentiated/moderately differentiated; PD/UD, poorly differentiated/undifferentiated; miR, microRNA; TNM, tumor node metastasis.

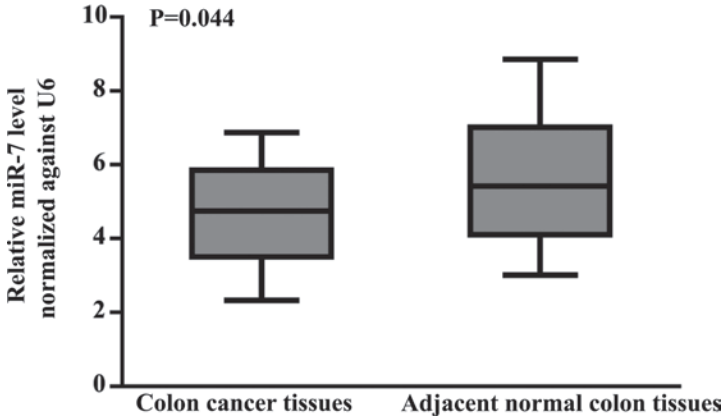

Figure 1. Expression of miR-7 in colon cancer. The expression level of miR-7 in colon cancer and adjacent normal colon tissues (n=60) was determined using reverse transcription-quantitative polymerase chain reaction. The expression levels of miR-7 are demonstrated as box plots, normalized against the levels of U6. The error bars represent a 95% confidence interval. miR, microRNA.

were captured at 0, 24 and 48 h after wounding using a phase contrast microscope (IX711; Olympus, Tokyo, Japan). The wound-healing areas were first compared to the wound area at 0 h, and were finally compared to the mock control using Image J 1.48u software (National Institutes of Health, MA, USA).

Statistical analysis. All data were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The data are presented as the mean ± standard deviation. The differences between the groups were assessed by Student's t-test or one-way analysis of variance. Pearson's correlation coefficient was used to assess the correlation between the expression levels of FAK and miR-7 in CC. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-7 is downregulated in CC and correlates with clinical significance. A total of 60 cases of fresh specimens of CC and adjacent normal colon tissues were used to detect the expression of miR-7 by RT-qPCR. The expression of miR-7 was markedly downregulated in CC compared with paired normal colon tissues (P=0.044; Fig. 1). As shown in Table I, the expression of miR-7 negatively correlated with lymph node metastasis (r=-2.29; P=0.022) and tumor node metastasis (TNM) stages (r=-2.698; P=0.007), regardless of gender, age and histopathologic differentiation of the patients with CC. These results suggested that miR-7 may be a potential tumor suppressor and may be important in the development of CC.

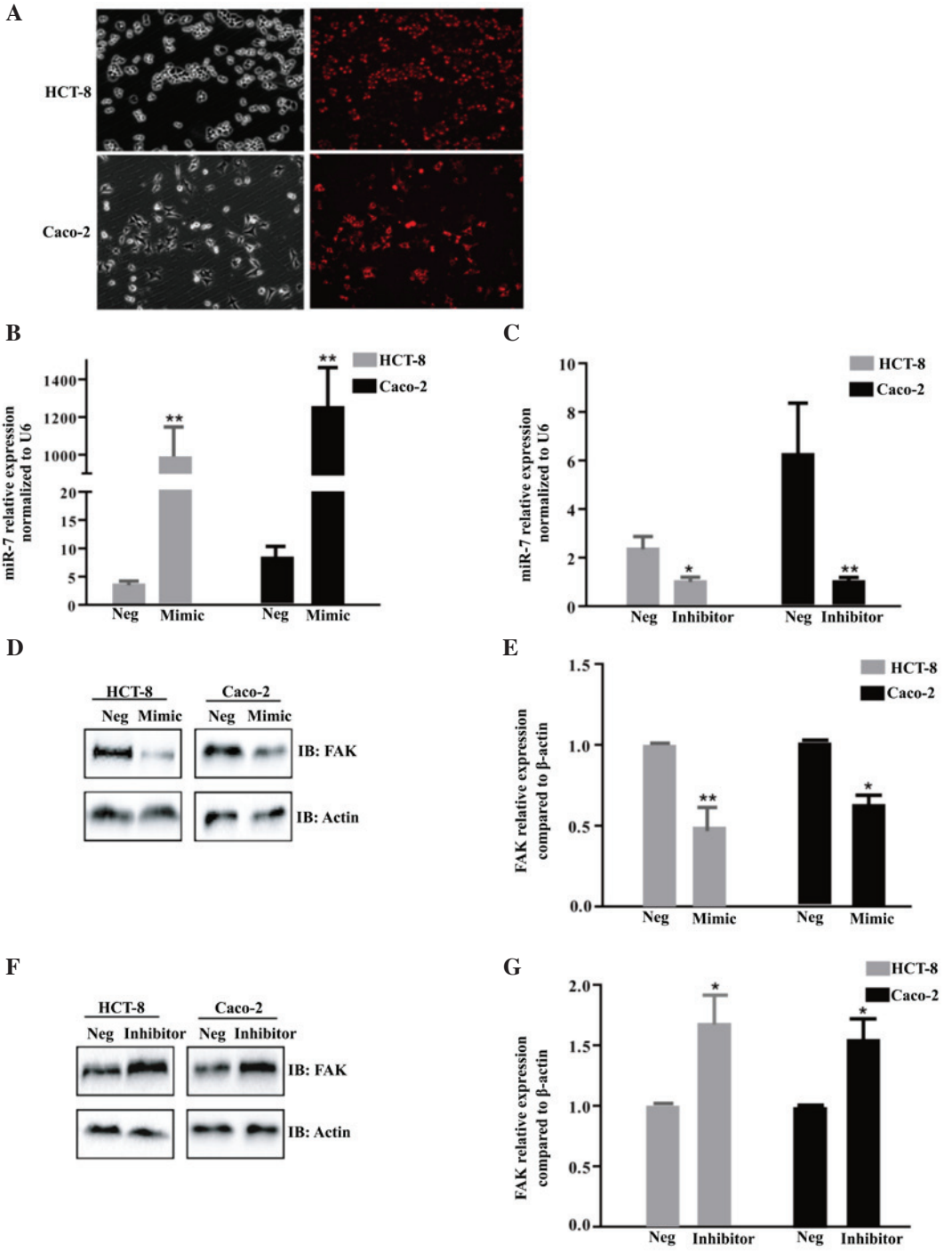

Figure 2. Alteration of the expression of miR-7 changed the protein expression of FAK. (A) Fluorescence images of Caco-2 and HCT-8 cells treated with the miR-7 mimic, negative-5'-cy3. The expression of miR-7 in HCT-8 and Caco-2 cells treated with (B) miR-7 mimic or (C) miR-7 inhibitor was determined by reverse transcription-quantitative polymerase chain reaction. (D) The expression of FAK was detected in HCT-8 and Caco-2 cells following transfection with the miR-7 mimic by western blotting. (E) Histograms illustrate the expression of FAK following transfection with the miR-7 mimic. The data are expressed as the mean ± standard deviation of three independent experiments (*P<0.05 and **P<0.01, compared with the Neg cells). (F) The expression of FAK was detected in HCT-8 and Caco-2 cells following transfection with the miR-7 inhibitor by western blotting. (G) Histograms illustrate the expression of FAK following transfection with the miR-7 inhibitor. The data are expressed as the mean ± standard deviation of three independent experiments (*P<0.05 and **P<0.01, compared with the Neg cells). FAK, focal adhesion protein; Neg, negative control; miR, microRNA; IB, immunoblotting.

Expression of miR-7 is associated with the protein expression of FAK. To further determine whether FAK was a downstream target of miR-7, miR-7m, miR-7i or NC were transfected into Caco-2 and HCT-8 cells. To confirm the transfection efficiency, fluorescence images were caputred 6 h after transfection using miR-7m, negative-5'-cy3 (Fig. 2A). Compared with the NC or mock, the expression of miR-7 was significantly increased or decreased following transfection with miR-7m or miR-7i, respectively in the HCT-8 and Caco-2 cells (Fig. 2B and C). The ectopic expression of miR-7 significantly decreased the protein expression of FAK, as determined by western blotting (P<0.05; Fig. 2D and E). However, a decrease in the expression

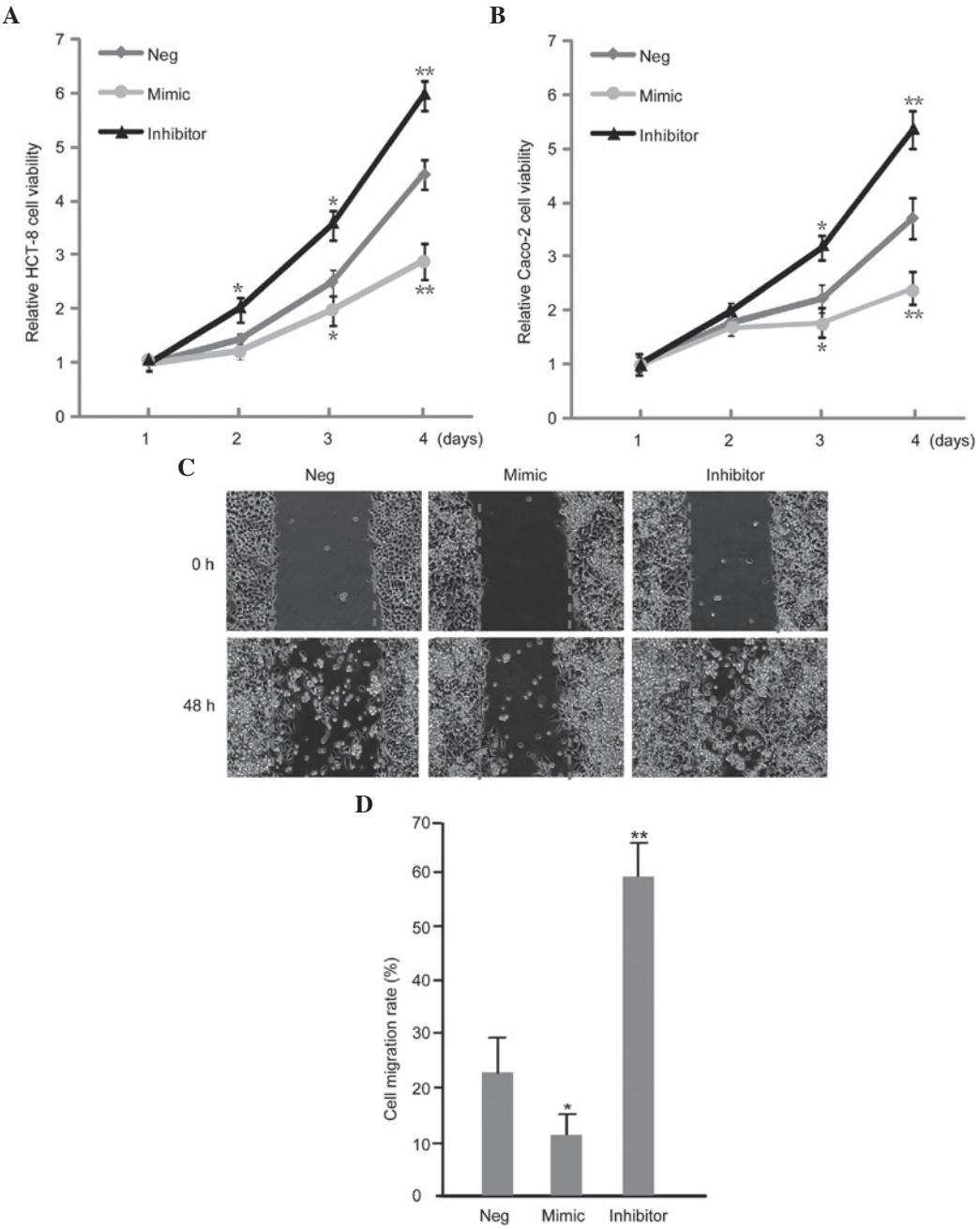

Figure 3. miR-7 suppresses the migration and proliferation of colon cancer cells. (A and B) Repression of miR-7 enhances the viability of colon cancer cells. Decreased viability was observed in gastric cancer cells transfected with miR-7 mimic. The cell viabilities of (A) HCT-8 and (B) Caco-2 cells were assessed by an MTT assay. The data are expressed as the mean ± standard deviation of three independent experiments (*P<0.05 and **P<0.01, compared with the Neg cells). (C and D) The migration of HCT-8 cells was reduced following transfection with miR-7 mimic, however, the repression of miR-7 accelerated the migration of HCT-8 cells, as determined by a wound healing assay. The data are expressed as the mean ± standard deviation of three independent experiments (*P<0.05 and **P<0.01, compared with the Neg cells). Neg, negative control; miR, microRNA.

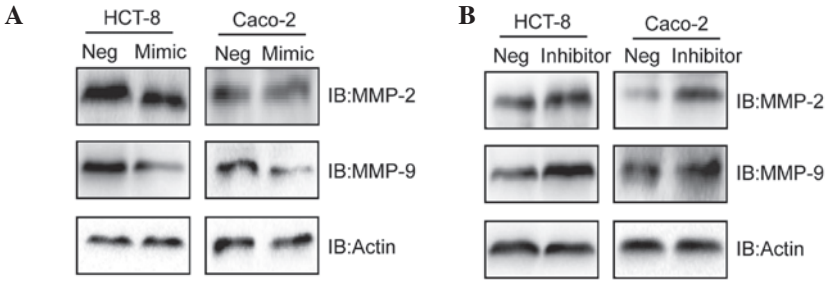

Figure 4. Effects of miR-7 on the MMP-2/MMP-9 pathway. (A) The expression levels of MMP2/MMP9 in HCT-8 and Caco-2 cells treated with the miR-7 mimic were determined by western blotting. (B) The expression levels of MMP2/MMP9 in HCT-8 and Caco-2 cells treated with miR-7 inhibitor were determined by western blotting. miR, microRNA; MMP, matrix metalloproteinase.

of miR-7 led to increased protein expression of FAK ($P<0.05$; Fig. 2F and G).

miR-7 influences the proliferation and migration of CC cells. The effect of miR-7 on the proliferation and migration of CC cells was assessed by a wound healing and MTT assay, respectively. It was revealed that the upregulation of miR-7 inhibited the proliferation and migration of HCT-8 cells (Fig. 3A and B). Additionally, downregulation of miR-7 enhanced the proliferation and migration of HCT-8 and Caco-2 cell lines (Fig. 3C and D).

miR-7 correlates with the expression levels of MMP-2 and MMP-9. Additionally, to investigate the mechanism underlying how miR-7 influences the biological behavior of CC cell lines, the levels of the invasion factors, MMP-2 and MMP-9, were assessed by western blotting following the up/downregulation of miR-7. Notably, it was revealed that the protein expression levels of MMP-2 and MMP-9 were significantly decreased/increased when HCT-8 and Caco-2 cells were transfected with miR-7m/i, respectively, as determined by western blotting (Fig. 4A and B).

Discussion

The incidence of CC revealed an increasing trend with recurrent cases causing liver metastasis (24). Previous studies suggested that metastasis is the predominant reason for the high mortality-associated with CC. In the human gene bank, >1,000 types of miRNAs are reported. Accumulating evidence suggested that miRNAs are important in CC tumorigenesis (25). At present, the correlation of CC with miRNAs remains to be elucidated. Balaguer *et al* (26) revealed that miR-137 acted as a tumor suppressor, however, was frequently silenced by promoter hypermethylation in colorectal carcinoma (CRC). Kulda *et al* (27) observed a higher expression of miR-21 and lower expression of miR-143 in CRC compared with adjacent normal colon tissue samples, which was associated with liver metastasis of CRC. Yamakuchi *et al* (28) suggested that miR-107 mediates the regulation of p53 on the hypoxic signaling and tumor angiogenesis pathways.

miR-7 is a member of the miRNA family, and is located on chromosome 15. The expression of miR-7 differs in various tumor types (29). Xiong *et al* (17) revealed that increased expression of miR-7 inhibits cell growth and invasion in the human A529 non-small cell lung cancer cell line. Notably, similar findings were observed in malignant glioma and schwannoma (30,31). FAK is a crucial signaling component and functions as a biosensor or integrator to control cell motility. Previous studies revealed that FAK, particularly phosphorylated (p)-FAK (Y397), was highly expressed in multiple tumor types, and was closely associated with the invasion and metastasis of the tumor (32). How FAK and its signaling pathway regulate the development of CC remains to be elucidated. Currently, research on FAK inhibitors is being performed to target its Y397 phosphorylation, for example, p-FAKY397 antibodies or FAK-related non-kinase (33), however the treatment remains limited. Whether there are more targets to control the invasion of CC by inhibiting the expression of FAK remains to be elucidated.

For the above issues, the present study aimed to investigate the effect of miR-7 on the biological behavior of CC and the correlation with FAK, which proved to be a promising target for the treatment and prognosis of CC.

Firstly, the present study revealed that the expression of miR-7 was decreased in CC tissues compared with the paired adjacent normal tissues, by RT-qPCR, and this correlated with lymph node metastasis and TNM stages in CC. Next, the expression of miR-7 was investigated *in vitro* and revealed that miR-7 inhibited the proliferation and invasiveness in CC cell lines using MTT and wound healing assays, respectively. Finally, it was revealed that the protein expression levels of FAK, MMP-2 and MMP-9 were significantly reduced following transfection with the miR-7m, however, increased following transfection with the miR-7i in CC cell lines.

These results were consistent with the results of a previous study revealing that miR-7 inhibited the growth and metastasis of glioma by targeting a negative regulator of FAK (14). miR-7 inhibited the epithelial-mesenchymal transition to suppress the growth and metastasis of breast cancer by targeting the protein expression of FAK (34).

In conclusion, the present study revealed that miR-7 suppressed the growth and proliferation of CC cell lines by targeting FAK. Notably, the present study provided novel insights into the mechanisms underlying tumor growth and metastasis. miR-7 may be a potential therapeutic strategy for the treatment of CC in the future.

Acknowledgements

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