# miR-197 promotes the invasion and migration of colorectal cancer by targeting insulin-like growth factor-binding protein 3

NA ZHOU[1], ZHAO SUN[1], NINGNING LI[1], YUPING GE[1], JIANFENG ZHOU[1], QIN HAN[2], LIN ZHAO[1] and CHUNMEI BAI[1]

[1]Department of Oncology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730; [2]Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing 100005, P.R. China

Received October 24, 2017; Accepted July 17, 2018

DOI: 10.3892/or.2018.6640

**Abstract.** The incidence and mortality of colorectal cancer (CRC) have been rising rapidly in China. A number of miRNAs have been confirmed to be involved in diverse biological processes of CRC. However, whether miR-197 plays a role in migration and invasion of CRC has never been explored. In the present study Transwell chambers were used in *in vitro* migration and invasion assays. Dual-luciferase reporter assay was employed to confirm the target of miR-197. RT-PCR and IHC staining were performed to quantify miR-197 and IGFBP3 expression, respectively. Clinicopathological features were collected for statistical analysis. We observed that the overexpression of miR-197 significantly promoted migration and invasion in 3 CRC cell lines including HCT8, HCT116 and SW480 (P<0.05), while the inhibition of miR-197 weakened both biological processes (P<0.05). In bioinformatics and dual-luciferase reporter assay, luciferase activities of IGFBP3-WT-transfected cells significantly decreased upon miR-197 overexpression and this inhibitory effect was abolished when miR-197 binding region in IGFBP3 3'-UTR was mutated, which indicated that miR-197 directly suppressed the expression of IGFBP3 in CRC cells by targeting its 3'UTR. Downregulation of the expression of IGFBP3 by using targeted siRNA led to significant enhancement of cell migration and invasion in two CRC cell lines including HCT8 and HCT116 (P<0.05). Finally, in cancerous tissues of CRC patients, the miR-197 level was inversely correlated with the expression of IGFBP3 (P=0.026), which indicated that miR-197 may modulate cell migration and invasion by targeting IGFBP3 in CRC patients. In conclusion, we revealed that miR-197 modulates IGFBP3 and therefore plays a critical role in regulating CRC migration and invasion.

*Correspondence to:* Professor Lin Zhao or Professor Chunmei Bai, Department of Oncology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Shuaifuyuan, Wangfujing, Dongcheng, Beijing 100730, P.R. China
E-mail: wz20010727@aliyun.com
E-mail: baichunmei196403@aliyun.com

*Key words:* miR-197, colorectal cancer, IGFBP3, migration, invasion

## Introduction

Colorectal cancer (CRC) is the most frequently diagnosed malignancy of the gastrointestinal tract. During recent decades, the incidence and mortality of CRC have been rapidly rising in China and the burden of this cancer will continue to be severe in the future (1,2). In spite of treatment combinations including surgery and adjuvant therapies, more than half of the cases with resected CRCs will finally result in tumor recurrence and metastasis. Current clinicopathological biomarkers are still incapable of accurately predicting tumor progression or further stratifying CRC patients with the same TNM stage. Therefore, it is necessary to develop new prognostic biomarkers for improving the stratification of CRC cases and for exploring original therapeutic alternatives.

MicroRNAs (miRNAs) are ~22 nucleotide, endogenous, non-coding RNAs that can induce translational suppression and mRNA degradation by binding to complementary sequences in the 3'untranslated regions (3'UTR) of the targeted mRNAs. Several studies revealed abundant miRNA expression abnormalities in CRC tissues compared with normal tissues. A number of miRNAs were proven to be involved in diverse biological processes of CRC such as cell proliferation, apoptosis, angiogenesis, invasion and metastasis. For example, the miR-143/145 cluster suppresses CRC cell growth through modulation of the KRAS signaling pathway (3,4). A positive feedback regulatory network exists between p53 and miR-34a, and the latter indirectly upregulates p53-dependent apoptosis in CRC (5,6). The c-Myc-mediated transcriptional suppression of miR-15-16 in hypoxia promotes tumor angiogenesis and hematogenous metastasis by upregulating FGF2 (7). Furthermore, a double-negative feedback loop between the miR-200 family and E-cadherin transcriptional suppressors ZEB1 and ZEB2 mediates epithelial-mesenchymal transition

(EMT), subsequently regulating the metastatic behavior of CRC (8).

Growing evidence suggests miR-197 as a novel biomarker for various cancers (9). Several studies revealed that miR-197 may be an oncomiR in cancer cell invasion and metastasis. For example, miR-197 induces epithelial-mesenchymal transition in pancreatic cancer cells along with the downregulation of p120 catenin, an E-cadherin interacting protein (10). By targeting CD82, miR-197 may also play a vital role as an invasion and migration promoter in gastric cancer and hepatocellular cancer via the EGFR-ERK1/2-MMP7 signaling pathway (11,12). Our previous research revealed that miR-197 could influence the sensitivity of CRC cells to fluorouracil (5-FU) by modulating the expression of thymidylate synthase (TYMS); however it had no impact on cell proliferation and cell cycle regulation (13). However, it is unclear whether miR-197 exhibits other biological functions in CRC. Thus, in the present study, we intended to uncover the role of miR-197 in cell migration and invasion of CRC and the possible underlying mechanisms.

## Materials and methods

*Tumor cell culture*. HCT8 (ileocecal colorectal adenocarcinoma), HCT116 (colorectal carcinoma) and SW480 (Dukes' type B, colorectal adenocarcinoma) cell lines were purchased from the Cell Resource Center, Institute of Basic Medical Sciences (IBMS), Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS/PUMC) (Beijing, China). They were maintained in Gibco™ Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (both from Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified 5% $CO_2$/95% air atmosphere at 37˚C.

*miRNA and siRNA transfection*. The synthetic miR-197 mimic, miR-197 inhibitor, mimic control and inhibitor control were purchased from GenePharma Inc. (Shanghai, China), and their sequences are listed in Table I. The siRNAs for IGFBP3 and siRNA control were synthesized (Invitrogen; Thermo Fisher Scientific, Inc.) and the sequences are listed in Table II.

*In vitro migration and invasion assays*. Transwell chambers (8-$\mu$m pore size; Costar; Corning Costar, Cambridge, MA, USA) were used in the *in vitro* migration assay. Colorectal cancer cells were transfected with the miR-197 mimic or the mimic control. Following 48 h, cells were detached with trypsin, washed with PBS and resuspended in serum-free medium. Two hundred microliters of cell suspension ($1x10^5$ cells/ml for miR-197 mimic and its control, $2x10^5$ cells/ml for miR-197 inhibitor and its control) was added to the upper chamber, and 500 ml of complete medium was added to the bottom well. The cells that had not migrated were removed from the upper surfaces of the filters using cotton swabs, and the cells that had migrated to the lower surfaces of the filters were fixed with 4% paraformaldehyde solution and stained with crystal violet. Images of 3 random fields (x10 magnification) were captured from each membrane, and the number of migratory cells was counted as previously described (14). Similar inserts coated with Matrigel were used to determine the invasive potential.

*RNA reverse transcription and RT-qPCR*. Total RNA was extracted using the Invitrogen™ TRIzol total RNA isolation reagent (Thermo Fisher Scientific, Inc.) and purified with the Column DNA Erasol kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. mRNA levels were assessed with RT-qPCR using SYBR-Green I (Takara Biotechnology Co., Ltd., Dalian, China). The gene expression level was normalized to the endogenous reference gene GAPDH. The experiments were performed in triplicate. The primers for RT-qPCR are listed in Table III. The primers for miR-197 and U6 were purchased from Qiagen (Duesseldorf, Germany) (Table IV). Reverse transcription of miRNAs was performed with a miScript Reverse Transcription kit (Qiagen). The expression of mature miRNAs was determined using miRNA-specific quantitative RT-qPCR (Takara Biotechnology Co., Ltd.). The expression levels were normalized to the U6 endogenous control and assessed by the comparative Ct ($\Delta\Delta$Cq) method (15).

*Prediction for target genes using bioinformatics web server.* Since miRNAs commonly act on posttranscriptional regulation by targeting 3'UTRs of mRNAs of target genes, in order to ascertain the mechanism underlying the involvement of miR-197 in biological processes, we first searched for possible target genes of miR-197 in the database on the web server 'Targetscan' (http://www.targetscan.org/vert_71/). Subsequently, we determined the candidate genes for our study from this repertoire of target genes by carefully reviewing corresponding literature and selecting the most possible target genes which had been reported to participate in cell migration, invasion and tumor metastasis.

*Dual-luciferase reporter gene construct and dual-luciferase reporter assay*. Fragments from 3 candidate genes including ADAMTS5, CBL and IGFBP3 whose 3'-UTRs contained the predicted binding site for hsa-miR-197, and flanking sequences on each side were synthesized with a short extension containing cleavage sites for *Xho*I (5'-end) and *Not*I (3'-end) (Table V); second fragments containing mutated binding site sequences were also synthesized. The two constructs were termed WT (Gene-wild type) and MT (Gene-mutant). The fragments were cloned into the psiCHECK™-2 vector (Promega Corporation, Madison, WI, USA). Ten nanograms of WT, MT or control vector and 200 nmol/l miR-197 mimic were transfected into 293T cells (293T cells were supplied by the Cell Center of Institue of Basic Medical Sciences, Chinese Academy of Medical Sciences) using Invitrogen™ Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cells were harvested 24 h following transfection and assayed for *Renilla* and firefly luciferase activities using the Dual-Luciferase Reporter Assay System (Promega Corporation).

*Western blot analysis*. Following washing twice with PBS, cells were lysed in ice-cold Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Nanjing, China) and manually scraped from the culture plates. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, electroblotted onto a polyvinylidene difluoride (PVDF)

Table I. Sequences of microRNAs.

| Name | Sequences |
|---|---|
| miR-197 mimic | 5'-UUC ACC ACC UUC UCC ACC CAG C-3'<br>5'-UGG GUG GAG AAG GUG GUG AAU U-3' |
| Mimic control | 5'-UUC UCC GAA CGU GUC ACG UTT-3'<br>5'-ACG UGA CAC GUU CGG AGA ATT-3' |
| miR-197 inhibitor | 5'-GCUGGGUGGAGAAGGUGGUGGUGAA-3' |
| Inhibitor control | 5'-CAG UAC UUU UGU GUA GUA CAA-3' |

Table II. IGFBP3 siRNA sequence.

| Name | Sense (5'-3') | Antisense (5'-3') |
|---|---|---|
| IGFBP3 siRNA | GUUGACUACGAGUCUCAGATT | UCUGAGACUCGUAGUCAACTT |

Table III. Primers (mRNA) for real-time PCR.

| Name | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| GAPDH | GAAGGTGAAGGTCGGAGTC | AAGATGGTGATGGGATTTC |
| IGFBP3 | GAGGGCGACACTGCTTTTTC | CCAGCTCCAGGAAATGCTAG |

Table IV. Primers (miRNA) for real-time PCR.

| miRNA | Primer sequence (5'-3') |
|---|---|
| hsa-miR-197 | TTCACCACCTTCTCCACCCAGC |
| U6 | CAAGGATGACACGCAAATTCG |

membrane and incubated with anti-IGFBP3 antibody (1:1,000 dilution; cat. no. sc-135947; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-GAPDH antibody (1:1,000 dilution; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.), followed by incubation with a secondary anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody (1:1,000; Zhongshan Golden Bridge Biotechnology, Beijing, China). Antibody-antigen complexes were detected using a chemiluminescent ECL reagent (EMD Millipore, Billerica, MA, USA).

*Clinical samples*. Fresh frozen samples of colorectal adenocarcinomas were collected from 21 patients who underwent surgery at Peking Union Medical College Hospital (PUMCH) from May 2015 to July 2015. All patients had signed written informed consents prior to enrolling in the study before admission. The use of human subjects in this study was also approved by the Ethics Committee at Peking Union Medical College Hospital (Beijing, China). This cohort includes 11 males and 10 females patients with average age of 60.5 (46-72 years). Basic clinicopathological features of these 21 patients are listed in Tables VI and VII.

*Immunohistochemical staining (IHC)*. IGFBP3 expression was evaluated by IHC staining. Briefly, after 5-$\mu$m sections were deparaffinized, antigen retrieval was performed by use of heat-induced epitoperetrieval with 10 mM citrate buffer. Sections were incubated with a monoclonal anitbody against IGFBP3 (cat. no. sc-135947 Santa Cruz Biotechnology, Inc.) at 1:250 dilution. The IGFBP3 antibody was detected using the avidin-biotin-peroxidase technique (Dako LSAB kit; Dako, Carpinteria, CA, USA). The expression levels of IGFBP3 were determined by a pathologist. The classification of '- and +' was defined by the percentage of IGFBP3 positive cells at the level of <5 and ≥5%, respectively.

*Statistical analysis*. Comparisons between different groups in migration and invasion assays were analyzed using t-tests (two-sided). ANOVA followed by Dunnette's test was applied to compare intergroup differences in luciferase reporter assays. Correlation between miR-197 and IGFBP3 expression was determined by correlate bivariate Spearman assay. Chi-square Fisher's exact test were used to calculate differences of miR-197 expression between different groups of clinicopathological factors. All data were calculated using SPSS 19.0 (IBM Corp., Armonk, NY, USA) and two-sided P-value <0.05 was considered to indicate a statistically significant difference.

## Results

*miR-197 enhances migration and invasion in CRC cell lines in vitro*. We first evaluated the efficacy of miR-197 overexpression and inhibition using synthetic miR-197 mimic, miR-197

Table V. Sequences of target genes and mutated target genes.

| Target genes | Sequences |
|---|---|
| IGFBP3 wild-type | F 5'-TCGAGCAGCTGGCTACAGCCTCGATTTATATTTCTGTTTGTGGTGAACTGATTTTTTTT AAACC AAAGTTTAGAAAGAGGGC-3' |
| | R 5'-GGCCGCCCTCTTTCTAAACTTTGGTTTAAAAAAAATCAGTTCACCACAAACAGAAAT ATAAATCGAGGCTGTAGCCAGCTGC-3' |
| IGFBP3 mutated | F 5'-TCGAGCAGCTGGCTACAGCCTCGATTTATATTTCTGTTTCTGATTTTTTTTAAACCAAA GTTAGAAAGAGGGC-3' |
| | R5'-GGCCGCCCTCTTTCTAAACTTTGGTTTAAAAAAAATCAGAAACAGAAATATAAATCGA GGCTGTAGCCAGCTGC-3' |
| ADAMTS5 wild-type | F 5'-TCGAGCAAAGATAACTGGAGGATTCAGCACTGATGCAGTCGTGGTGAACAGGAGGT CTACCTAACGCACAGAAAGTCATGCTTGC-3' |
| | R5'-GGCCGCAAGCATGACTTTCTGTGCGTTAGGTAGACCTCCTGTTCACCACGACTGCATC AGTGCTGAATCCTCCAGTTATCTTTGC-3' |
| ADAMTS5 mutated | F 5'-TCGAGCAAAGATAACTGGAGGATTCAGCACTGATGCAGTCCAGGAGGTCTACCTAAC GCACAGAAAGTCATGCTTGC-3' |
| | R 5'-GGCCGCAAGCATGACTTTCTGTGCGTTAGGTAGACCTCCTGGACTGCATCAGTGCTG AATCCTCCAGTTATCTTTGC-3' |
| CBL wild-type | F 5'-TCGAGAGCTATGAGGCACCTCCTACGTCTGTTTTCTGGCTGTGGTGACTTGGGATTTT TAACCTTATATATCTTTTTCCTTTGC-3' |
| | R 5'-GGCCGCAAAGGAAAAAGATATATAAGGTTAAAAATCCCAAGTCACCACAGCCAGAA AACAGACGTAGGAGGTGCCTCATAGCTC-3' |
| CBL mutated | F 5'-TCGAGAGCTATGAGGCACCTCCTACGTCTGTTTTCTGGCTCTTGGGATTTTTAACCTTA TATATCTTTTTCCTTTGC-3' |
| | R 5'-GGCCGCAAAGGAAAAAGATATATAAGGTTAAAAATCCCAAGAGCCAGAAAACAGAC GTAGGAGGTGCCTCATAGCTC-3' |

F, forward; R, reverse.

Table VI. Relationship of miR-197 and IGFBP3 expression with clinicopathological factors.

| Clinicopathological features | miR-197 high expression ΔCT level (≤7.64) n (%) | miR-197 low expression ΔCT level (>7.64) n (%) | P-value | IGFBP3 Negative n (%) | IGFBP3 Positive n (%) | P-value |
|---|---|---|---|---|---|---|
| Tumor invasion | | | 0.090 | | | 0.131 |
| T2-3 | 7 (41.2) | 10 (58.8) | | 9 (52.9) | 8 (47.1) | |
| T4 | 4 (100) | 0 (0) | | 4 (100.0) | 0 (0%) | |
| N staging | | | 0.635 | | | 0.336 |
| N1 | 4 (66.7) | 2 (33.3) | | 5 (83.3) | 1 (16.7) | |
| N2 | 7 (46.7) | 8 (53.3) | | 8 (53.3) | 7 (46.7) | |
| Histology | | | 0.149 | | | 0.325 |
| Good and moderate | 10 (62.5) | 6 (37.5) | | 11 (66.8) | 5 (31.2) | |
| Poor | 1 (20.0) | 4 (80.0) | | 2 (40.0) | 3 (60.0) | |
| Lymphovascular invasion | | | 0.890 | | | 0.776 |
| No | 8 (53.3) | 7 (46.7) | | 9 (60.0) | 6 (40.0) | |
| Yes | 3 (50.0) | 3 (50.0) | | 4 (66.7) | 2 (33.3) | |
| Site | | | 0.659 | | | 0.965 |
| Colon | 6 (46.2) | 7 (53.8) | | 8 (61.5) | 5 (38.5) | |
| Rectum | 5 (62.5) | 3 (37.5) | | 5 (63.5) | 3 (37.5) | |

Table VII. Clinicopathological features of the CRC patients.

| No. | Histology | Site | Stage | miR-197 (ΔCt) | IGFBP3 (-/+) |
|---|---|---|---|---|---|
| 1 | Adenocarcinoma | Rectum | IIIB (pT3N1) | 7.69 | Positive |
| 2 | Adenocarcinoma | Colon | IIIB (pT3N1) | 7.69 | Positive |
| 3 | Adenocarcinoma | Rectum | IIIC (pT4N2b) | 7.38 | Negative |
| 4 | Adenocarcinoma | Rectum | IIIB (pT3N1) | 6.57 | Negative |
| 5 | Adenocarcinoma | Colon | IIIB (pT3N1) | 8.68 | Positive |
| 6 | Adenocarcinoma | Rectum | IIIB (pT3N1) | 7.72 | Positive |
| 7 | Adenocarcinoma | Colon | IIIB (pT3N2a) | 8.77 | Negative |
| 8 | Adenocarcinoma | Colon | IIIB (pT4aN1) | 2.20 | Negative |
| 9 | Adenocarcinoma | Rectum | IIIA (pT2N1) | 7.84 | Positive |
| 10 | Adenocarcinoma | Colon | IIIB (pT3N1) | 5.70 | Negative |
| 11 | Adenocarcinoma | Colon | IIIB (pT3N2a) | 4.34 | Negative |
| 12 | Adenocarcinoma | Colon | IIIB (pT3N1) | 7.94 | Positive |
| 13 | Adenocarcinoma | Rectum | IIIB (pT3N1) | 6.54 | Negative |
| 14 | Adenocarcinoma | Colon | IIIC (pT3N2b) | 2.37 | Negative |
| 15 | Adenocarcinoma | Colon | IIIB (pT3N1) | 8.75 | Positive |
| 16 | Adenocarcinoma | Colon | IIIB (pT3N2a) | 8.47 | Negative |
| 17 | Adenocarcinoma | Rectum | IIIB (pT4aN1) | 6.95 | Negative |
| 18 | Adenocarcinoma | Rectum | IIIB (pT4aN1) | 7.64 | Negative |
| 19 | Adenocarcinoma | Colon | IIIB (pT3N1) | 7.99 | Negative |
| 20 | Adenocarcinoma | Colon | IIIB (pT3N2a) | 7.22 | Positive |
| 21 | Adenocarcinoma | Colon | IIIB (pT3N1) | 7.00 | Negative |

inhibitor and corresponding controls in CRC cell lines (Fig. 1). We then investigated the effect of miR-197 on the migration and invasion in colorectal cell lines. HCT8, HCT116 and SW480 cells were transfected with either miR-197 mimic, mimic control, miR-197 inhibitor or inhibitor control and then subjected to cell migration and invasion assays (Fig. 2). In the HCT8 cell line, significantly more tumor cells in the miR-197 mimic group had migrated to the lower surface of the filter compared with the miR-197 mimic control group (33.50±4.65 vs. 18.00±3.91, P<0.05) in the migration assay and similarly, significantly more cells invaded to the lower surface in the miR-197 mimic group than its control group in the invasion assay (24.75±2.21 vs. 18.00±3.91, P<0.05, Fig. 2A). On the contrary, significant less HCT8 cells in miR-197 inhibitor group were found on the lower surface of the filter compared with its control group in the migration assay (32.25±2.98 vs. 47.25±3.20, P<0.05) and also in the invasion assay (31.00±3.16 vs. 46.75±4.42, P<0.05, Fig. 2A). Furthermore, similar results were also achieved in the HCT116 and SW480 cells (Fig. 2B and C), indicating that miR-197 could regulate migration and invasion in these 3 CRC cell lines *in vitro*.

*miR-197 directly suppresses the expression of IGFBP3 by targeting its 3'UTR*. The mRNAs which may be targeted by miR-197 were predicted, and genes associated with tumor invasion and metastasis were selected. According to this principle, we screened out 3 candidate target genes including IGFBP3, ADAMTS5 and CBL. Predicted miR-197 binding sites within the 3'UTR of mRNAs of these 3 genes are listed in Figs. 3A, 4B and D, respectively.

Compared to the control group, miR-197 mimic significantly decreased the luciferase activity when co-transfected with IGFBP3-WT reporter plasmid (Fig. 3B, P<0.05). However, this inhibitory effect on luciferase activity mediated by miR-197 mimic was abolished by mutant reporter plasmid (Fig. 3B, P<0.05). In addition, miR-197 overexpression had no effect on the luciferase activities of ADAMTS5 (P=0.974, Fig. 4A) and CBL (P=0.098, Fig. 4C). We decided to choose IGFBP3 as research target of our study due to its tight connection with CRC according to multiple studies.

To demonstrate whether miR-197 could exert a similar influence on IGFBP3 expression in CRC, we transfected HCT8 cell line again with the miR-197 mimic, miR-197 inhibitor or their corresponding controls, and then determined IGFBP3 expression at the RNA and protein levels, respectively. The protein level of IGFBP3 in HCT8 cells was obviously decreased following transfection with miR-197 mimic and increased following transfection with miR-197 inhibitor (Fig. 3C), while the alterations of IGFBP3 mRNA did not reach statistical significance upon overexpression and inhibition of miR-197 (P>0.05, Fig. 3D). These findings revealed that miR-197 suppressed the protein output of IGFBP3 rather than degrading its mRNA, indicating that IGFBP3 was regulated by miR-197 in CRC cells at the post-transcriptional level.

*Downregulation of IGFBP3 boosts migration and invasion of CRC cell lines*. We then investigated the role of IGFBP3 in CRC cell migration and metastasis. Small interfering RNA (siRNA) designed to target IGFBP3 was employed. The transfection of siRNA resulted in a significant reduction in IGFBP3 mRNA

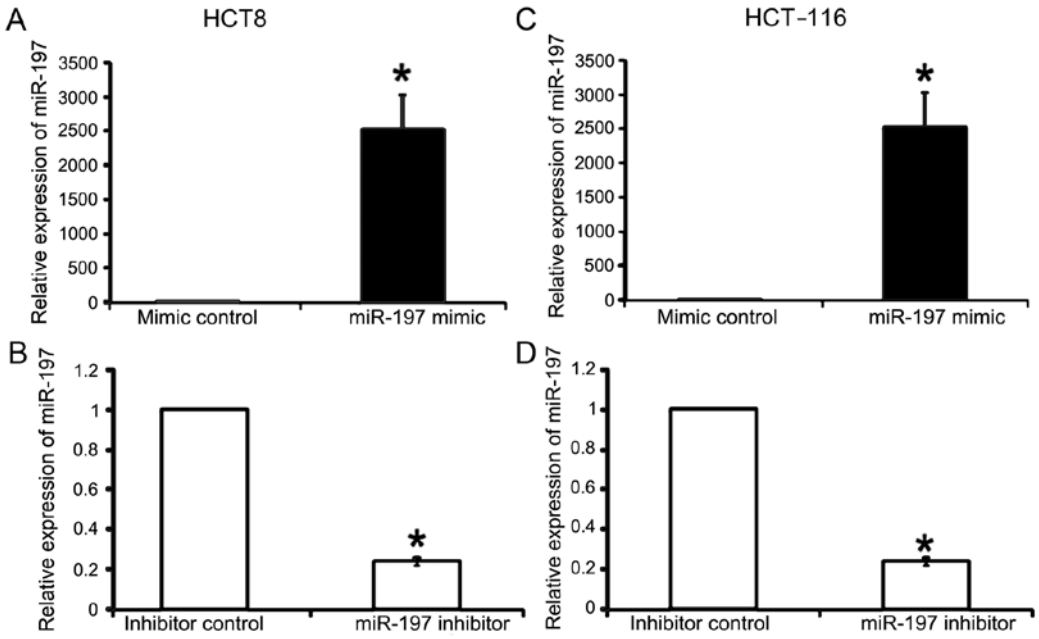


Figure 1. Transfection efficiency of miR-197 mimic, miR-197 inhibitor and their controls in CRC cells by RT-qPCR. (A and B) In HCT8 cells and (C and D) HCT-116 cells, miR-197 expression was significantly increased in cells transfected with miR-197 mimics compared with its control and miR-197 expression was significantly reduced in cells transfected with miR-197 inhibitor compared with its control. Data are from 3 independent experiments. *P<0.05.

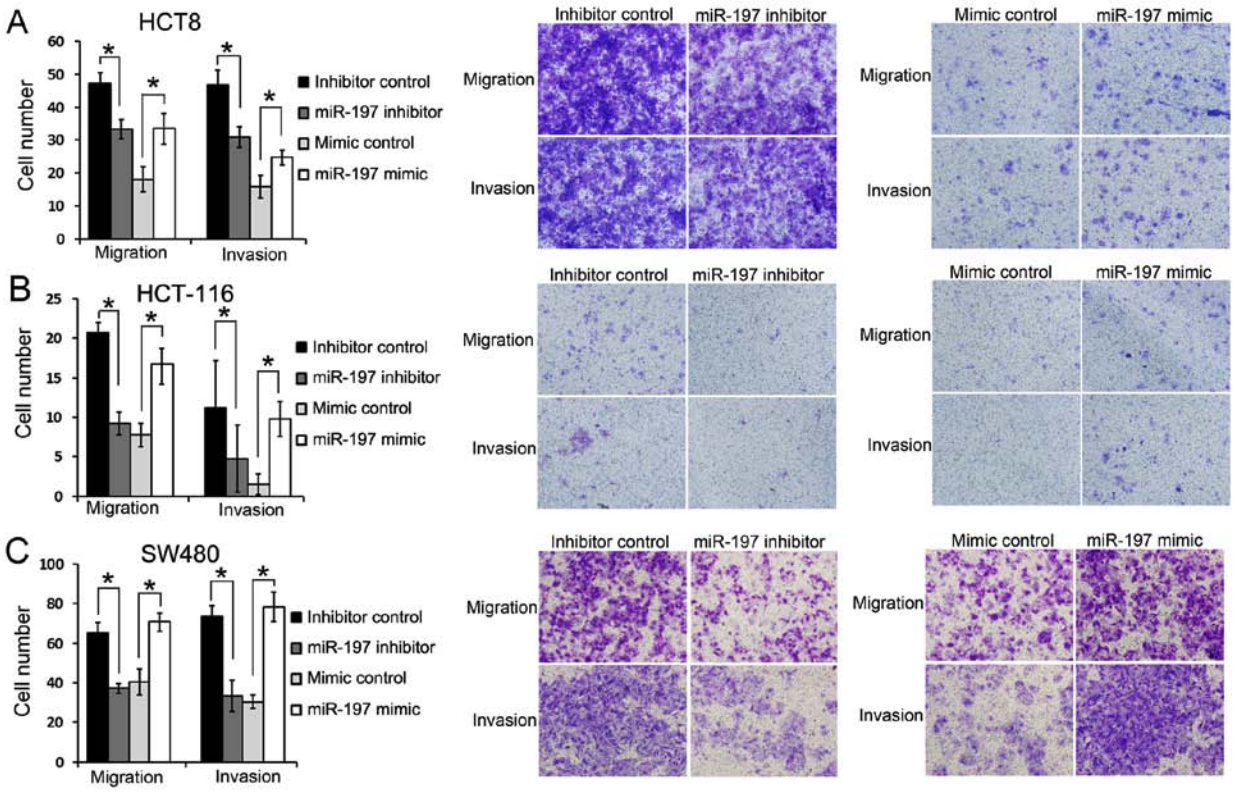


Figure 2. (A) The effect of miR-197 expression on the migration and invasion in HCT8 cells. Transwell migration (n=4) and invasion (n=4) assays revealed that HCT8 cells transfected with the miR-197 inhibitor had significantly lower invasive and migratory potentials than the control. On the contrary, HCT8 cells transfected with the miR-197 mimic had greater invasive and migratory potentials than the control. *P<0.05. (B and C) The effect of miR-197 expression on the migration and invasion in HCT-116 and SW480 cells, respectively: HCT-116 and SW480 cells transfected with the miR-197 inhibitor had lower invasive and migratory potentials than the control. Conversely, HCT-116 and SW480 cells transfected with the miR-197 mimic had greater invasive and migratory potentials than the control. *P<0.05.

and protein level in HCT8 and HCT-116 cells compared to the siRNA-control (Fig. 5). Both cell lines were transfected with either IGFBP3 siRNA (si-IGFBP3) or siRNA-control, and then subjected to cell migration and cell invasion assay.

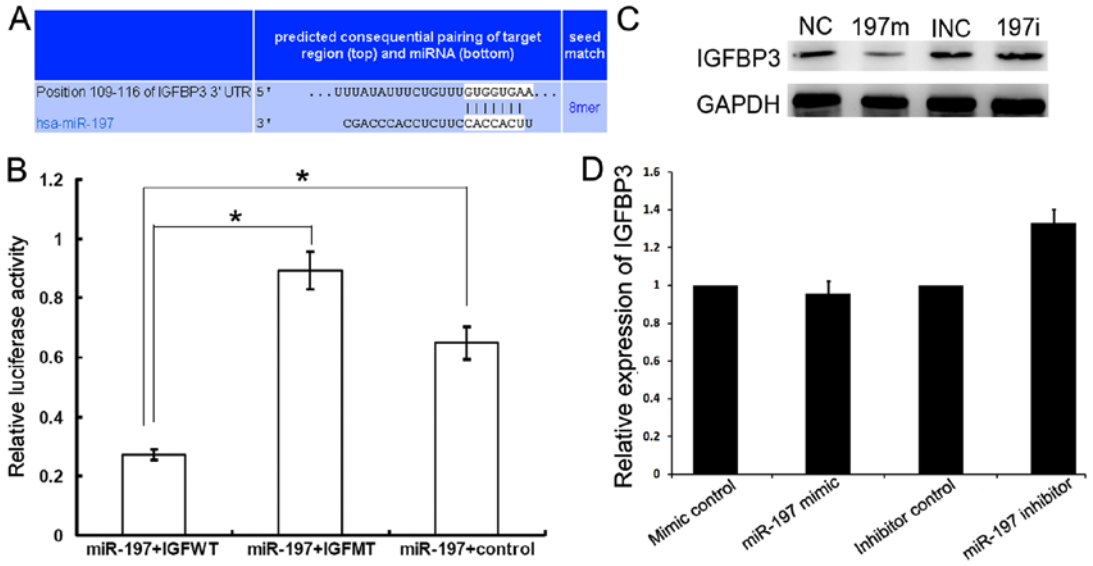


Figure 3. (A) Analysis of the 3'UTR sequence of the IGFBP3 gene by TargetScan revealed a putative miR-197 binding site. (B) The miR-197 binding site on IGFBP3-3'UTR was confirmed by luciferase assay in 293T cells (n=3). The luciferase activities of cells co-transfected with wild-type IGFBP3 3'UTR sequence (IGFWT) decreased significantly upon miR-197 overexpression compared to those of cells co-transfected with the control vector (*P<0.05) or mutant IGFBP3 3'UTR sequence (IGFMT) (*P<0.05). Luciferase activity of IGFMT was not significantly affected by miR-197 compared to the control group (P>0.05). NC, negative control; 197m, miR-197 mimic; INC, negative control inhibitor; 197i, miR-197 inhibitor. (C) Western blot assay showed that IGFBP3 protein expression was regulated by miR-197 in HCT8 cells. (D) Real-time PCR showed that the mRNA level of IGFBP3 was not significantly affected by miR-197.

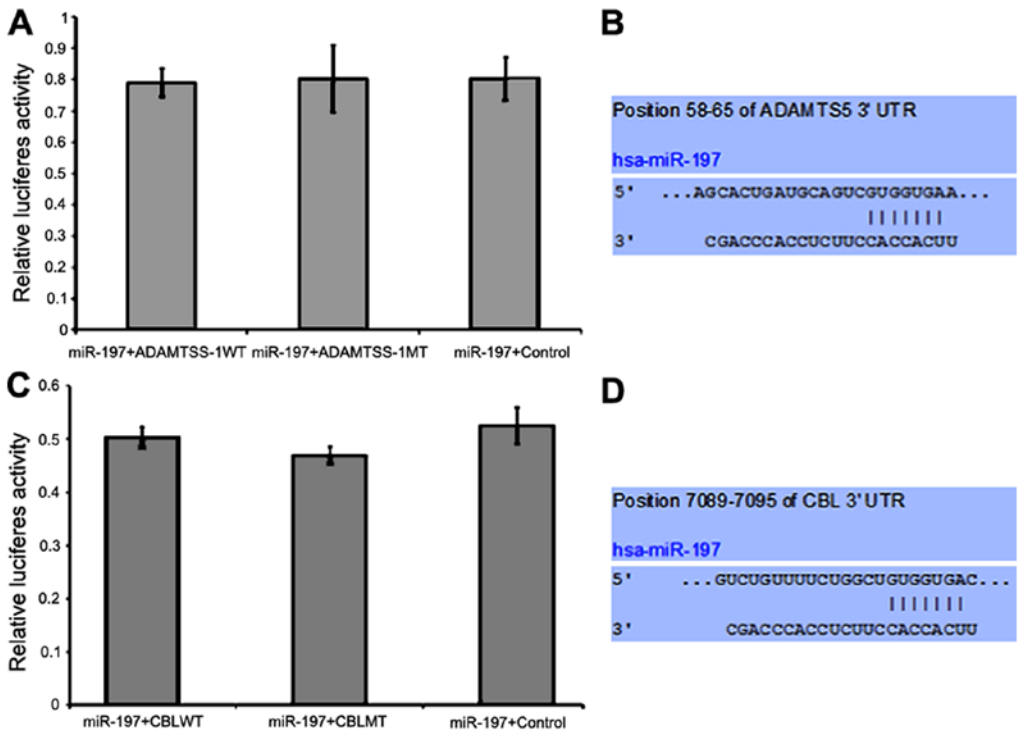


Figure 4. The binding of miR-197 to the 3'UTR of ADAMTS5 and CBL genes are excluded by luciferase assay in 293T cells. (A and C) The luciferase activities of cells transfected with wild-type 3' UTR of ADAMTS5 (ADAMTS5-1WT) and CBL (CBLWT) upon miR-197 overexpression did not differ from those of cells transfected with the control vector or mutant 3'UTRs. Data represent the mean ± SD of 3 independent experiments. (B and D) Analysis of the 3'UTR sequences of ADAMTS5 and CBL by TargetScan revealed putative miR-197 binding sites.

In the migration assay, the number of migratory HCT8 cells in the si-IGFBP3 group was significantly greater than the migratory HCT8 cells in the si-control group (54.75±4.57 vs. 32.75±7.18, P<0.05, Fig. 6A and B). In addition, significantly more HCT8 cells in the si-IGFBP3 group had invaded to the lower surface of filters compared with those in si-control group (12.75±3.50 vs. 2.75±1.70, P<0.05) in the invasion assay (Fig. 6A and B). Similar results were also achieved in HCT-116 cells (Fig. 6C and D). Cell motility of both assays was significantly enhanced following IGFBP3 downregulation

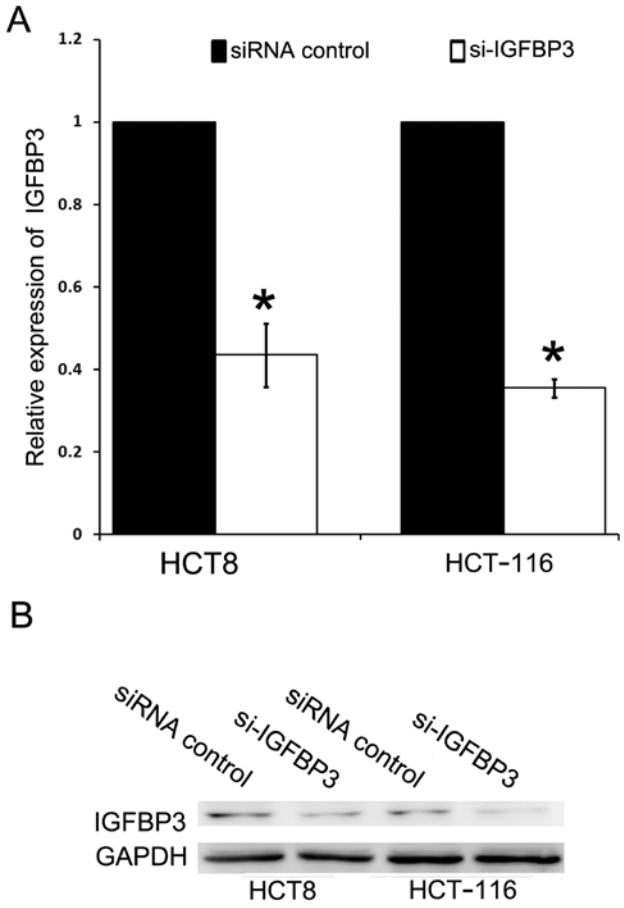


Figure 5. Efficiency of siRNA-mediated deletion of IGFBP3. (A) RT-PCR assay revealed the IGFBP3 expression at the mRNA level was significantly reduced in HCT8 and HCT-116 cells transfected with si-IGFBP3 compared with those transfected with siRNA control. *P<0.05; (B) Western blot assay revealed the IGFBP3 expression at the protein level in HCT8 and HCT-116 cells following transfection with si-RNA control and si-IGFBP3.

compared with the control in HCT-116 cells, with similar results in HCT8 cells, which indicated that IGFBP3 was a suppressor protein in the process of migration and invasion in CRC cells.

*Correlation of miR-197 with IGFBP3 in CRC patients*. We assessed the miR-197 quantity of cancer tissues using RT-PCR in fresh frozen samples and IGFBP3 expression using IHC in corresponding paraffin samples in order to analyze the correlation between miR-197 and IGFBP3 expression in CRC patients. In this cohort of 21 stage III CRC patients, there were one stage IIIA cancer, 2 stage IIIC cancers, and 18 patients with stage IIIB cancer; 13 patients had colon cancer and 8 patients had rectum cancer. The median ΔΔCq value of miR-197 was 7.64 (2.2-8.77). We dichotomized IGFBP3 protein expression using 'positive' or 'negative' as displayed in Fig. 7. There were 8 IGFBP3-positive and 13 IGFBP3-negative tumors. We revealed that the miR-197 ΔΔCq level was significantly associated with the expression of IGFBP3 with statistical significance (P=0.026), so that miR-197 expression was negatively related to IGFBP3 protein expression in the CRC tumor tissues.

Furthermore, we divided miR-197 expression level into two groups using the median ΔΔCq value (≤7.64 vs. >7.64), and explored whether there was any difference in miR-197 expression between the different groups of clinicopathological factors. Although we discovered a trend that there were more miR-197 high expression tumors in the T4 group compared to the T2-3 group, it was not statistically significant (P=0.09), and thus were other factors such as lymph node stage, histology, lymphovascular involvement and tumor site (Table VI). In addition we did not detect any differences in the expression of IGFBP3 between different groups of pathological features (Table VI). Finally, we aimed to clarify the association between prognosis and differential expression of miR-197 plus IGFBP3, but we failed due to the limited events of recurrence and death. Basic clinical information of this cohort of CRC patients is listed in Table VII.

## Discussion

The functions of miR-197 in tumorigenesis are complicated. Several studies have found that miR-197 plays controversial roles in the initiation and progression of different malignancies. It decreases apoptosis in p53 wild-type non-small cell lung cancer (NSCLC) (16), but has the opposite function in multiple myeloma (17). It inhibits proliferation in glioblastoma (18) but promotes invasion and metastasis in gastric (11), pancreatic (10) and hepatocellular cancer (HCC) (12). In colorectal cancer, our previous studies revealed that miR-197 had no influence on proliferation and cell cycle distribution, but it mediated the 5-FU response by targeting thymidylate synthase in stage IV CRC (13). In the present study, we

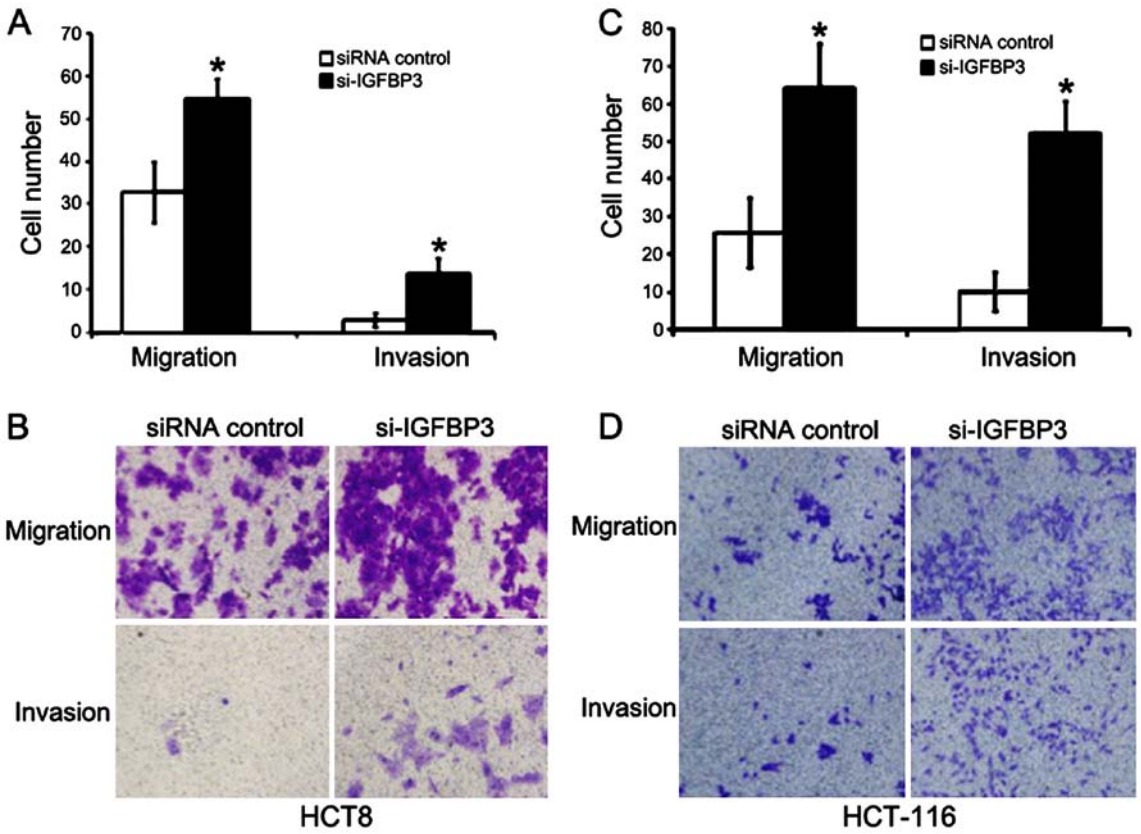


Figure 6. Efficacy of IGFBP3 RNA interference on cell migration and invasion in CRC cells. (A and B) Transwell migration (n=4) and invasion (n=4) assays revealed that HCT8 cells transfected with the IGFBP3 siRNA (200 nM) had greater invasive and migratory potentials than the control (siRNA control). n=4, *P<0.05. (C and D) Transwell migration (n=4) and invasion (n=4) assays showed that HCT-116 cells transfected with the IGFBP3 siRNA (200 nM) had greater invasive and migratory potentials than the control (siRNA control). n=4, *P<0.05.

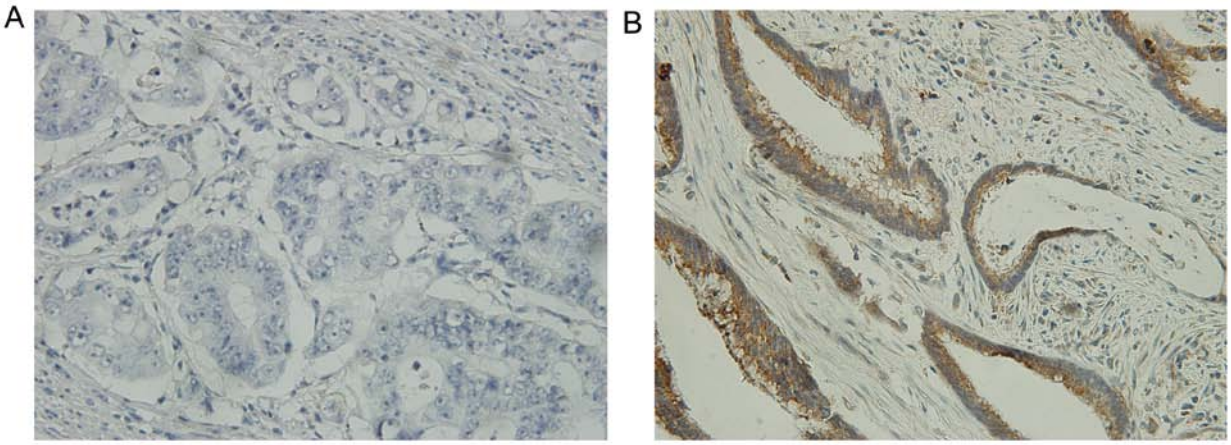


Figure 7. Immunohistochemistry (IHC) images of differential IGFBP3 expression. For exploring the association between IGFBP3 and miR-197 in colorectal cancer tissue, we dichotomized IGFBP3 protein expression using IHC. (A) Negative staining was defined as the staining of <5% target cells; (B) positive staining was defined as the staining of ≥5% target cells.

observed a unanimous phenomenon in 3 different CRC cell lines that miR-197 upregulation promoted migration and invasion, while its downregulation hindered the same process. This phenomenon in CRC parallels that observed in other malignancies arising from the epithelium. To the best of our knowledge, we revealed for the first time the biological activities of miR-197 and the mechanism underlying its function in CRC.

Several studies have revealed that miR-197, alone or grouped with other miRNAs, may be a novel prognostic biomarker for several human tumors that arise from epithelial tissues. For example, miR-197 was found to be an unfavorable prognostic or metastatic predictor in NSCLC, and to be associated with larger tumors and squamous histology (19). Finally, meta-analysis displayed that a panel of multiple miRNAs may deliver much greater predicting efficacy than a single miRNA

assay (20) and recently, 7 miRNA signatures including miR-197 have been presented to be a feasible survival indicator for CRC patients (21), which collaborates our assumption about the role of miR-197 in CRC prognosis. In order to clarify whether miR-197 may also regulate IGFBP3 in CRC tissues similar to the results we achieved *in vitro*, we detected the miR-197 level and protein expression of IGFBP3 in tumor tissues of 21 CRC patients, and discovered a negative association between miR-197 and IGFBP3. However, we failed to detect any differences in the miR-197 expression level between the different groups of clinicopathological characteristics. This may be due to the fact that our cohort was small and highly homogenous with stage III cancers. However a tendency of a greater proportion of miR-197 high expression tumors was observed in the T4 group compared to the T2-3 group, which may hint that the deeper the tumor invades, the more miR-197 is expressed. However, direct evidence of the association of miR-197 with disease-free survival and overall survival was not obtained, due to the fact that the majority of these CRC cases were not presented with disease recurrence and cancer-related death; therefore, there were not enough data to come to a conclusion about the effect of miR-197 on prognosis.

In the present study, we confirmed IGFBP3 as one of the targets of miR-197 and their inverse correlation in CRC patient samples. IGFBP3 is the most abundant IGFBP in circulation. It attenuates IGF1-induced IGFR1 signaling events by sequestering IGF1 away from IGF1R (22). To date, accumulating evidence revealed that the IGF1/IGF1R axis stimulates epithelial to mesenchymal transition and contributes to tumor metastasis including CRC (23,24). In addition, IGFBP3 may also affect migration and invasion in a complex intrinsic network, an IGFR-independent manner. For example, IGFBP3 inhibits intracellular adhesion in head and neck squamous cell carcinoma through blocking c-jun and c-fos transcription and integrin β4 expression (25). It also suppresses prostate cancer progression through degrading NF-κB signaling (26). In CRC, IGFBP3 has long been considered as a tumor suppressor (27). However, the biological role and mechanism of IGFBP3 in the progression and metastasis in CRC are poorly understood. We used *in vitro* assays and found that IGFBP3 decreased migration and invasion in the HCT8 and HCT-116 cell lines. It is not known yet whether this inhibitory effect correlates with disease progression, resembling the situation in breast cancer (28). Thus far, the cellular effects of IGFBPs were reported to be mediated post-translationally. Other than methylation and proteolysis, miRNA regulation may be another mode for IGFBP3 inactivation. For example, miR-21 enhances glioblastoma tumorigenesis by downregulating IGFBP3 (29); onco-miRs miR-155 and miR-125b also target IGFBP3, promoting migration and invasion in HCC and NSCLC through the IGF-II/IGF-1R axis (30) and downstream PI3K/AKT activation (31). miRNAs regulate complementary mRNAs by inducing translational suppression and mRNA destablization. Although IGFBP3 mRNA decay accounts for the majority of miRNA-mediated regulations in the above-mentioned studies, we speculated that miR-197 targets IGFBP3 to promote invasion and migration in CRC through a translational suppression manner, since the IGFBP3 mRNA level was only slightly altered while its protein expression was significantly suppressed upon miR-197 expression. One comparable scenario is the regulation of proliferation and invasion in CRC by miR-21 through the PTEN/PI-3K/Akt signaling pathway (32). Dissociation of translation initiation complex eIF4F was thought to be a mechanism underlying miRNA-mediated translational suppression (33). Reactivation of suppressed targets by degradation of RISC under certain contexts could also explain why target mRNA does not have to end up in degradation (34). However, future studies are needed to uncover how two silencing modes of miRNAs contribute to the overall silencing of target mRNAs.

Due to its critical role in the IGF1/IGF1R axis and various bioactivity, emerging clinical studies have attempted to apply the serum IGFBP3 level and IGFBP3 methylation status for prognostic prediction in CRC. An elevated serum IGFBP3 level was previously reported to be correlated with a significant reduction of CRC mortality (35). Recently, high IGFBP3 gene methylation in the primary tumor has been identified as an independent predictor for recurrence risk in stage II CRC (36). However, other studies using the same methodologies revealed quite contrary or insignificant results (37,38). These data indicated that the aforementioned IGFBP3 assessments may not be accurate reflections of the local situation or IGFBP3 may be regulated by an intricate system. We used IHC staining to evaluate local IGFBP3 protein expression in tumor tissues but still no associations were found with clinicopathological factors. Nevertheless, it is too soon to exclude local IGFBP3 protein expression from being a biomarker. In gastric cancer, IGFBP3 deficiency in IHC was found to be correlated with advanced lymph node stage and poor survival (39). It is still necessary and promising to probe the relationship of local IGFBP3 expression with other clinicopathological factors and CRC patient prognosis in a larger cohort with mature survival data.

In addition, we also explored two other possible genes targeted by miR-197 using luciferase reporter assay and found that miR-197 overexpression had no inhibitory effect on the luciferase activity of CBL and ADAMTS5. Therefore, we excluded these two genes as targets of miR-197. Attention should be drawn to the fact that we did not succeed in cloning and expressing full-length IGFBP3 because it is too large, which may compromise the role of IGFBP3 as a tumor suppressor in CRC cell lines and its relationship with miR-197 from the opposite angle.

In conclusion, in the present study, we revealed that miR-197 overexpression could boost migration and invasion in CRC cell lines. miR-197 directly regressed the expression of IGFBP3 in CRC cells through targeting its 3'UTR.

**Acknowledgements**

The authors thank all the patients who participated in this study.

**Funding**

The present study was supported by grants from the National Natural Science Foundation of China (nos. 81472785 and 61435001) and grants from The 2016 PUMCH Science Fund for Junior Faculty (Pumch-2016-1.13).

## Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

## Authors' contributions

NZ and ZS co-conceived the study, managed its design and drafted the manuscript. NL and YG participated in acquisition and analysis of clinical data. JZ and QH contributed to the design of the study and carried out the molecular biological assay. LZ and CB participated in revising the manuscript critically for important intellectrual content. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee at Peking Union Medical College Hospital (Beijing, China). All patients signed written informed consents prior to enrolling in the present study before admission.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References


1. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132, 2016.
2. Zhang Y, Shi J, Huang H, Ren J, Li N and Dai M: Burden of colorectal cancer in China. Zhonghua Liu Xing Bing Xue Za Zhi 36: 709-914, 2015 (In Chinese).
3. Chen X, Guo X, Zhang H, Xiang Y, Chen J, Yin Y, Cai X, Wang K, Wang G, Ba Y, *et al*: Role of miR-143 targeting KRAS in colorectal tumorigenesis. Oncogene 28: 1385-1392, 2009.
4. Mendell JT and Olson EN: MicroRNAs in stress signaling and human disease. Cell 148: 1172-1187, 2012.
5. Yamakuchi M, Ferlito M and Lowenstein CJ: miR-34a repression of SIRT1 regulates apoptosis. Proc Natl Acad Sci USA 105: 13421-13426, 2008.
6. Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, Zhai Y, Giordano TJ, Qin ZS, Moore BB, *et al*: p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr Biol 17: 1298-1307, 2007.
7. Xue G, Yan HL, Zhang Y, Hao LQ, Zhu XT, Mei Q and Sun SH: c-Myc-mediated repression of miR-15-16 in hypoxia is induced by increased HIF-2α and promotes tumor angiogenesis and metastasis by upregulating FGF2. Oncogene 34: 1393-1406, 2015.
8. Bracken CP, Gregory PA, Kolesnikoff N, Bert AG, Wang J, Shannon MF and Goodall GJ: A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res 68: 7846-7854, 2008.
9. Wang DD, Chen X, Yu DD, Yang SJ, Shen HY, Sha HH, Zhong SL, Zhao JH and Tang JH: miR-197: A novel biomarker for cancers. Gene 591: 313-319, 2016.
10. Hamada S, Satoh K, Miura S, Hirota M, Kanno A, Masamune A, Kikuta K, Kume K, Unno J, Egawa S, *et al*: MiR-197 induces epithelial-mesenchymal transition in pancreatic cancer cells by targeting p120 catenin. J Cell Physiol 228: 1255-1263, 2013.
11. Xu L, Hou Y, Tu G, Chen Y, Du YE, Zhang H, Wen S, Tang X, Yin J, Lang L, *et al*: Nuclear Drosha enhances cell invasion via an EGFR-ERK1/2-MMP7 signaling pathway induced by dysregulated miRNA-622/197 and their targets *LAMC2* and *CD82* in gastric cancer. Cell Death Dis 8: e2642, 2017.
12. Dai W, Wang C, Wang F, Wang Y, Shen M, Chen K, Cheng P, Zhang Y, Yang J, Zhu R, *et al*: Anti-miR-197 inhibits migration in HCC cells by targeting KAI 1/CD82. Biochem Biophys Res Commun 446: 541-548, 2014.
13. Sun Z, Zhou N, Han Q, Zhao L, Bai C, Chen Y, Zhou J and Zhao RC: MicroRNA-197 influences 5-fluorouracil resistance via thymidylate synthase in colorectal cancer. Clin Transl Oncol 17: 876-883, 2015.
14. Sun Z, Han Q, Zhou N, Wang S, Lu S, Bai C and Zhao RC: Micro RNA-9 enhances migration and invasion through KLF17 in hepatocellular carcinoma. Mol Oncol 7: 884-894, 2013.
15. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25: 402-408, 2001.
16. Fiori ME, Barbini C, Haas TL, Marroncelli N, Patrizii M, Biffoni M and De Maria R: Antitumor effect of miR-197 targeting in p53 wild-type lung cancer. Cell Death Differ 21: 774-782, 2014.
17. Yang Y, Li F, Saha MN, Abdi J, Qiu L and Chang H: miR-137 and miR-197 induce apoptosis and suppress tumorigenicity by targeting MCL-1 in multiple myeloma. Clin Cancer Res 21: 2399-2411, 2015.
18. Tian LQ, Liu EQ, Zhu XD, Wang XG, Li J and Xu GM: MicroRNA-197 inhibits cell proliferation by targeting GAB2 in glioblastoma. Mol Med Rep 13: 4279-4288, 2016.
19. Mavridis K, Gueugnon F, Petit-Courty A, Courty Y, Barascu A, Guyetant S and Scorilas A: The oncomiR miR-197 is a novel prognostic indicator for non-small cell lung cancer patients. Br J Cancer 112: 1527-1535, 2015.
20. Wang R, Wen H, Xu Y, Chen Q, Luo Y, Lin Y, Luo Y and Xu A: Circulating microRNAs as a novel class of diagnostic biomarkers in gastrointestinal tumors detection: A meta-analysis based on 42 articles. PLoS One 9: e113401, 2014.
21. Xu M, Kuang Y, Wang M, Han X and Yang Q: A microRNA expression signature as a predictor of survival for colon adenocarcinoma. Neoplasma 64: 56-64, 2017.
22. Firth SM and Baxter RC: Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23: 824-854, 2002.
23. Li H, Batth IS, Qu X, Xu L, Song N, Wang R and Liu Y: IGF-IR signaling in epithelial to mesenchymal transition and targeting IGF-IR therapy: Overview and new insights. Mol Cancer 16: 6, 2017.
24. Yao C, Su L, Shan J, Zhu C, Liu L, Liu C, Xu Y, Yang Z, Bian X, Shao J, *et al*: IGF/STAT3/NANOG/Slug signaling axis simultaneously controls epithelial-mesenchymal transition and stemness maintenance in colorectal cancer. Stem Cells 34: 820-831, 2016.
25. Lee HJ, Lee JS, Hwang SJ and Lee HY: Insulin-like growth factor binding protein-3 inhibits cell adhesion via suppression of integrin β4 expression. Oncotarget 6: 15150-15163, 2015.
26. Han J, Jogie-Brahim S, Harada A and Oh Y: Insulin-like growth factor-binding protein-3 suppresses tumor growth via activation of caspase-dependent apoptosis and cross-talk with NF-κB signaling. Cancer Lett 307: 200-210, 2011.
27. Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR and Kley N: Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature 377: 646-649, 1995.
28. Zielinska HA, Bahl A, Holly JM and Perks CM: Epithelial-to-mesenchymal transition in breast cancer: A role for insulin-like growth factor I and insulin-like growth factor-binding protein 3? Breast Cancer 7: 9-19, 2015.
29. Yang CH, Yue J, Pfeffer SR, Fan M, Paulus E, Hosni-Ahmed A, Sims M, Qayyum S, Davidoff AM, Handorf CR and Pfeffer LM: MicroRNA-21 promotes glioblastoma tumorigenesis by down-regulating insulin-like growth factor-binding protein-3 (IGFBP3). J Biol Chem 289: 25079-25087, 2014.
30. El Tayebi HM, Waly AA, Assal RA, Hosny KA, Esmat G and Abdelaziz AI: Transcriptional activation of the IGF-II/IGF-1R axis and inhibition of IGFBP-3 by miR-155 in hepatocellular carcinoma. Oncol Lett 10: 3206-3212, 2015.
31. Wang HH, Wang YC, Wu DW, Hung CS, Chen CY and Lee H: Targeting insulin-like growth factor-binding protein-3 by microRNA-125b promotes tumor invasion and poor outcomes in non-small-cell lung cancer. Tumour Biol 39: 1010428317694316, 2017.

32. Xiong B, Cheng Y, Ma L and Zhang C: MiR-21 regulates biological behavior thourgh the PTEN/PI-3K/Akt signalling pathway in human colorectal cancer cells. Int J Oncol 42: 219-228, 2013.
33. Iwakawa HO and Tomari Y: The functions of microRNAs: mRNA decay and translational respression. Trends Cell Biol 25: 651-665, 2015.
34. Kundu P, Fabian M, Sonenberg N, Bhattacharyya SN and Filipowicz W: HuR protein attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA. Nucleic Acids Res 40: 5088-5100, 2012.
35. Haydon AM, Macinnis RJ, English DR, Morris H and Giles GG: Physical activity, insulin-like growth factor 1, insulin-like growth factor binding protein 3, and survival from colorectal cancer. Gut 55: 689-694, 2006.
36. Fu T, Pappou EP, Guzzetta AA, Calmon Mde F, Sun L, Herrera A, Li F, Wolfgang CL, Baylin SB, Iacobuzio-Donahue CA, *et al*: *IGFBP-3* gene methylation in primary tumor predicts recurrence of stage II colorectal cancers. Ann Surg 263: 337-344, 2016.
37. Perez-Carbonell L, Balaguer F, Toiyama Y, Egoavil C, Rojas E, Guarinos C, Andreu M, Llor X, Castells A, Jover R, *et al*: *IGFBP3* methylation is a novel diagnostic and predictive biomarker in colorectal cancer. PLoS One 9: e104285, 2014.
38. Wolpin BM, Meyerhardt JA, Chan AT, Ng K, Chan JA, Wu K, Pollak MN, Giovannucci EL and Fuchs CS: Insulin, the insulin-like growth factor axis, and mortality in patients with nonmetastatic colorectal cancer. J Clin Oncol 27: 176-185, 2009.
39. Xue M, Fang Y, Sun G, Zhuo W, Zhong J, Qian C, Wang L, Wang L, Si J and Chen S: IGFBP3, a transcriptional target of homeobox D10, is correlated with the prognosis of gastric cancer. PLoS One 8: e81423, 2013.