miR-410 acts as an oncogene in colorectal cancer cells by targeting dickkopf-related protein 1 via the Wnt/β-catenin signaling pathway

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Received January 2, 2018; Accepted August 28, 2018

DOI: 10.3892/ol.2018.9710

Abstract. Colorectal cancer (CRC) is a common malignancy with high morbidity. MicroRNAs (miRNAs or miRs) have been demonstrated to be critical post-transcriptional regulators in tumorigenesis. The current study aimed to investigate the effect of miR-410 on the proliferation and metastasis of CRC. The expression of miR-410 was examined in CRC cell lines. SW-480 and HCT-116 CRC cell lines were employed and transfected with miR-410 inhibitor or miR-410 mimics. The association between miR-410 and dickkopf-related protein 1 (DKK-1) was verified by luciferase reporter assay. Cell viability and apoptosis were detected by Cell Counting Kit-8 (CCK-8) and flow cytometry assay. Cell migration and invasion capacity were determined by Transwell assay. The protein level of DKK1, β-catenin and phosphorylated glycogen synthase kinase-3β (pGSK-3β) were analyzed by western blotting. miR-410 was revealed to be upregulated in CRC cell lines. Further studies identified DKK-1 as a direct target of miR-410. In addition, knockdown of miR-410 promoted the expression of DKK, inhibited CRC cell proliferation, migration and invasion capacity, and induced cell apoptosis, while overexpression of miR-410 reversed these results. miR-410 silencing also decreased β-catenin and pGSK-3β levels. The current study indicated that miR-410 negatively regulates the expression of DKK-1 *in vitro*. miR-410 promotes malignancy phenotypes in CRC cell lines. This regulatory effect of miR-410 may be associated with the Wnt/β-catenin signaling pathway. Therefore, miR-410 could be used as a biomarker for predicting the progression of CRC.

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Key words: microRNA-410, dickkopf-related protein 1, colorectal cancer, proliferation, apoptosis, metastasis

Introduction

Colorectal cancer (CRC) is a common malignancy and the fifth leading cause of cancer-associated cases of mortality for males and females in China (1). The morbidity of CRC has increased rapidly in recent years and distant metastasis accounts for the majority of cancer-associated cases of mortality in patients with CRC (2). Evidence has suggested that the overall five-year survival rate for patients with metastatic CRC is approximately 10-15%, showing an unsatisfactory prognosis (3,4). Therefore, improved understanding of the molecular interactions that occur in the initiation and progression of CRC may be helpful in identifying therapeutic targets and providing new prognostic treatments.

MicroRNAs (miRNAs or miRs) are a type of non-coding single-strand RNA molecule with a length of 20-24 nucleotides (5). These molecules are endogenously synthesized and negatively regulate the expression of genes by binding to their 3'-untranslated-region (3'-UTR) (6). Numerous studies have reported that miRNAs are involved in a wide range of biological processes and aberrant expression of miRNAs is associated with tumorigenesis and progression. For example, miR-200a regulates the proliferation and metastasis of pancreatic cancer through modulating the DEK gene (7), miR-543 promotes metastasis of prostate cancer by binding to RKIP (8), and miR-33 is downregulated in breast cancer tissues and acts as a tumor suppressor by targeting HMGA2 (9).

It has also been reported that a number of miRNAs are involved in CRC progression and prognosis. Among them, miR-410 has been verified to be aberrantly expressed in several human malignant cancer types and may function as a tumor suppressor in endometrial cancer, myeloma, lung cancer and breast cancer (10-13). Certain studies have reported that miR-410 regulates biological functions of CRC cells by targeting FHL1, ITPKB and Bak1 (14-16). In the current study, it was confirmed that miR-410 functions as a tumor suppressor in CRC cells by suppressing cell proliferation, migration and invasion. Furthermore, the current study reported that miR-410 targets dickkopf-related protein 1 (DKK1) and elucidated the underlying mechanism of the miR-410/DKK1 axis in CRC. To the best of our knowledge, the current study is the first to

identify this finding and therefore may shed new light on the therapeutic strategies for CRC.

Materials and methods

Cell culture and transfection. The CRC cell lines SW-480, SW-620, HT-29, HCT-116 and normal colon epithelial cell line FHC were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Pan Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in humidified atmospheric conditions of 5% CO_2. For transfection, miR-410 mimics (AUC AUGAUGGGCUCCUCGGUGUACACCGAGGAGCCCAUC AUGAU), miR-410 inhibitors (UAGUACUACCCGAGGAGC CACAUGUGGCUCCUCGGGUAGUACUA) and a negative control inhibitor (NC inhibitor) were constructed by Biossci Biotechnology Co. (Wuhan, China, http://www.biossci.com/). Transfections were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The concentration of miR-410 mimics was 50 nmol/l and of miR-410 inhibitors was 100 nmol/l, following instructions from Biossci Biotechnology Co. Cells were harvested after 48 h for further analyses.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from SW-480 and HT-116 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA purity was determined by a DU800 UV/Vis Spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA) and 100 ng RNA was used for complementary DNA (cDNA) synthesis using the ReverTra Ace-α-kit (Toboyo Life Science, Osaka, Japan) following the manufacturer's protocol. qPCR was performed using SYBR Green Real-Time PCR Master mix (Toyobo Life Science). The expression of miR-410 and DKK1 mRNA was normalized to U6 and β-actin, respectively. PCR was performed with the following thermocycling conditions: Initial denaturation at 94˚C for 4 min, 40 cycles of 94˚C for 30 sec, 56˚C for 30 sec and 72˚C for 25 sec, using the ABI 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific Inc.), according to the manufacturer's protocols. The relative amount of miRNA or mRNA was calculated via the $2^{-\Delta\Delta Cq}$ method (17) and the primer sequences used are shown in Table I.

Cell viability assay. The viability of SW-480 and HT-116 cells was measured by a cell viability test, using Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Jiangsu, China). Cells were inoculated into 96-well plates at a density of $2x10^3$ cells/well for 24 h and then transfected with miR-410 inhibitor or NC inhibitor according to the aforementioned manufacturer's protocol. SW-480 and HT-116 cells were stained with 20 μl of CCK-8 reagent for 4 h before detecting the absorbance at 450 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, Inc.).

Cell apoptosis assay. Cell apoptosis was examined using the Annexin V-FITC/Propidium Iodide (PI) staining kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). SW-480 and HT-116 cells were seeded in 6-well plates at a density of 10^6 cells/ml. At 24 h after transfection, the cells were labeled with Annexin V-FITC for 15 min in the dark. A total of 50 μg/ml of PI was added to each sample for 30 min. Cell apoptosis distribution was analyzed to evaluate the percentage of apoptotic cells by flow cytometry using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell invasion and migration assays. Cell invasion and migration assays were performed using Transwell plates (Corning Life Sciences, NY, USA) with 8-μm-pore size membranes with Matrigel (for invasion assay) or without Matrigel (for migration assay). SW-480 and HT-116 cells were used at 48 h post-transfection with miR-410 inhibitor or NC inhibitor. Briefly, $3x10^4$ cells were seeded in the upper chamber while medium containing 10% FBS was placed in the lower chamber. After incubation at 37˚C for 24 h, cells on the upper chamber membrane were wiped away. Then, cells on the lower chamber membrane were stained with 0.2% crystal violet for 30 min. Five predetermined fields were counted under a Olympus BX50 light microscope (magnification, x100; Olympus Corporation, Tokyo, Japan). All assays were performed in triplicate.

Database prediction. To explore the association between miR-410 and DKK1, an *in silico* prediction was performed using open access software (TargetScan, http://www.targetscan.org; PicTarget, https://pictar.mdc-berlin.de/ and miRanda https://microrna.org). A putative binding site for miR-410 was identified within the 3'-UTR of DKK1.

Plasmid construction and luciferase reporter assays. The putative and mutated miR-410 target binding sequences in DKK1 were synthesized and cloned into luciferase reporters to generate the wild-type (DKK1-WT) or mutated-type (DKK1-MUT) reporter plasmids. The mutant 3'-UTR sequence of DKK1 was obtained using an overlap-extension PCR method (18). Then, the sequences including the predicted wild and mutant target sites were subcloned into a psiCHECK-2 vector (Promega Corporation, Madison, WI, USA), and validated by sequencing by Sangon Biotech Co., Ltd. (Shanghai, China). For the luciferase reporter assay, SW-480 cells were seeded at $1x10^5$ cells/well on a 24-well plate. The cells were then co-transfected with miR-410 inhibitor or NC inhibitor using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Cells were harvested at 48 h post-transfection and luciferase activities were compared with *Renilla* luciferase activity using a Dual-Luciferase Reporter Assay system (Promega Corporation).

Western blot analysis. Total cellular proteins were lysed using RIPA Buffer (Beyotime Institute of Biotechnology), followed by centrifugation at 15,000 x g for 20 min at 4˚C. A bicinchoninic acid assay (Beyotime Institute of Biotechnology) was performed to quantify protein concentrations. Briefly, equivalent amounts of protein of 30 μg per lane were resolved by 10% SDS-PAGE gel electrophoresis and subsequently blotted onto polyvinylidene difluoride membranes followed by blocking at 4˚C for 1 h with TBS containing 0.05% Tween-20 (TBST) buffer with 5% non-fat milk and incubation with the

Table I. Reverse transcription-quantitative polymerase chain reaction primer sequences.

Gene	Primer sequences (5'-3')
DKK1	F: AGTACTGCGCTAGTCCCACC
	R: TCCTCAATTTCTCCTCGGAA
miR-410	F: AAUAUAACACAGAUGGCCUGU
	R: CCGUGCUCGACUUUCCGGCG
U6 snRNA	F: CTCGCTTCGGCAGCACATATACT
	R: ACGCTTCACGAATTTGCGTGTC
GAPDH	F: TGAAGGTCGGTGTGAACGGATTTGGTC
	R: CATGTAGGCCATGAGGTCCACCAC

DKK1, dickkopf-related protein 1; miR, microRNA; snRNA, small nuclear RNA.

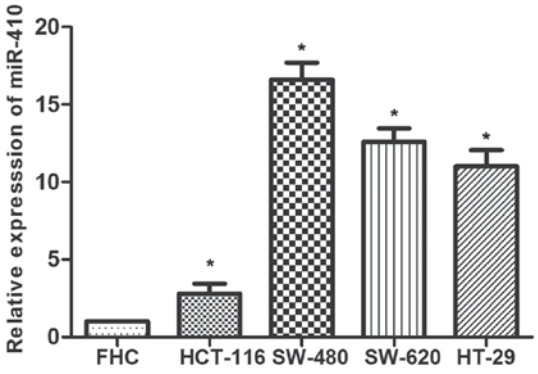

Figure 1. miR-410 expression in CRC cell lines. miR-410 expression measured by reverse transcription-quantitative polymerase chain reaction. *P<0.5 vs. FHC. CRC, colorectal cancer; miR, microRNA.

following primary antibodies: Anti-DKK1(dilution, 1:1,000; cat. no. ab109416); anti-β-catenin (dilution, 1:5,000; cat. no. ab32572); anti-GSK-3β (dilution, 1:5,000; cat. no. ab32391) (all from Abcam, Cambridge UK); and anti-phosphorylated glycogen synthase kinase-3β (p-GSK-3β) (dilution, 1:1,000; cat. no. D85E12; CST Biological Reagents Co., Ltd., Shanghai, China) at 4˚C overnight. GAPDH was used as a loading control. After washing with TBST buffer, membranes were incubated with goat anti-rabbit IgG antibody (dilution, 1:100; cat. no. LK2003; Sungene Biotech, Co., Ltd, Tianjin, China) at room temperature for 1 h. All bands were visualized with an ECL system kit [MultiSciences (Lianke) Biotech Co., Ltd, Hangzhou, China]. Densitometry was performed by ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All statistical analyses were performed using SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation. Differences were assessed by two-tailed Student's t-test, analysis of variance and a Student-Newman-Keuls post hoc test as appropriate. All experiments were performed at least three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-410 in CRC cell lines. To investigate whether miR-410 is involved in CRC development, miR-410 levels in four CRC cell lines (SW-480, SW-620, HT-29 and HCT-116) were examined by RT-qPCR. As shown in Fig. 1, the data demonstrated that miR-410 expression was significantly upregulated in CRC cell lines compared with the control. SW-480 and HT-116 cell lines were employed in subsequent experiments.

miR-410 induces CRC cell proliferation and inhibits cell apoptosis. To investigate the effect of miR-410 in CRC development, miR-410 inhibitor and NC inhibitor were used to evaluate the biological properties of miR-410 in SW-480 and HT-116 cells. Transfection efficiency was determined using RT-qPCR. As the results demonstrate, miR-410 expression was significantly inhibited in SW-480 cells and significantly upregulated in HT-116 cells following transfection with miR-410 inhibitor and miR-410 mimics, respectively (Fig. 2A). The results of the CCK-8 assay revealed that knockdown of miR-410 significantly inhibited cellular viability compared with NC group, while miR-410 overexpression markedly promoted cellular viability (Fig. 2B). In addition, flow cytometry data demonstrated that apoptotic rate of SW-480 was significantly increased by miR-410 inhibitor, while the apoptotic rate of HT-116 cells was suppressed by miR-410 mimics compared with NC group (Fig. 2C). The current data demonstrated that miR-410 is closely associated with cell proliferation and apoptosis in CRC cells.

miR-410 promotes cell migration and invasion of CRC cells. To delineate the role of miR-410 in the metastasis of CRC, a Transwell assay was employed to evaluate the migration and invasion capacity of CRC cells. It was identified that down-regulation of miR-410 significantly inhibited cell migration in SW-480 and HT-116 cells (Fig. 3A and B). Compared with NC inhibitor, the number of invaded cells was dramatically reduced when cells were treated with miR-410 inhibitor (Fig. 3C and D). These results suggest that miR-410 promotes the migration and invasion capacity of CRC cells.

miR-410 directly targets DKK1 and negatively regulates DKK1 expression. An open access database was employed to predict the putative binding sequences between miR-410 and DKK1. To further confirm whether DKK1 is a direct target of miR-410, the luciferase reporter vectors of the DKK1 3'-UTR containing miR-410 binding sites (DKK-1 WT), or a mutated version (DKK-MUT), were constructed. Co-transfection was performed with miR-410 inhibitor (or NC inhibitor) and DKK-1 WT (or DKK-1 MUT) plasmids into SW480 cells. A dual-luciferase reporter assay was used to measure the reporter activities of the different constructs. The results revealed that knockdown of miR-410 significantly increased reporter vector activity of DKK-1 3'-UTR in SW-480 cells but had no effect on the mutated reporter vector (Fig. 4A and B). To further explore the modulation of DKK1 expression by miR-410, western blot and RT-qPCR analyses were performed. It was demonstrated that miR-410 silencing led to an increase in

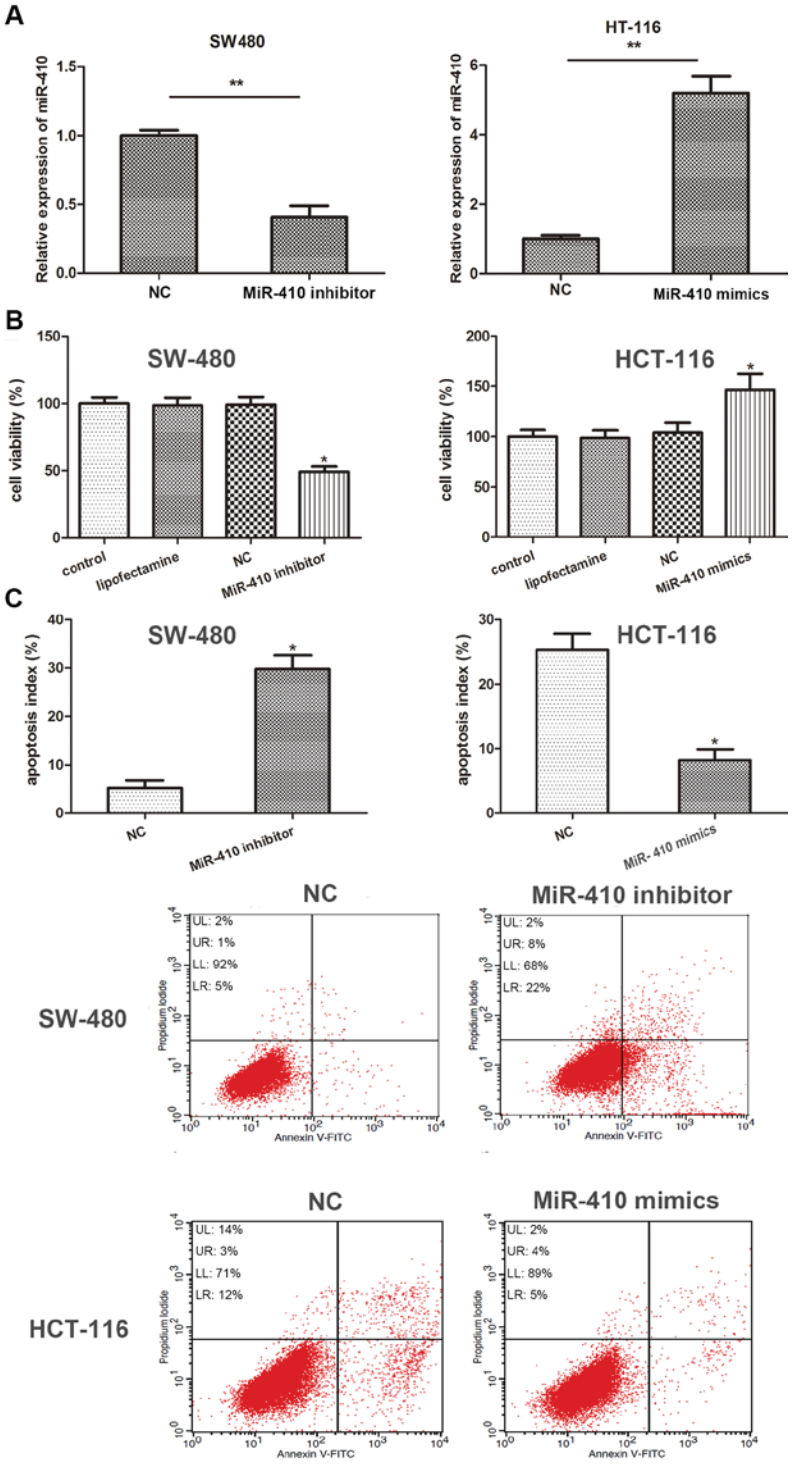

Figure 2. Effects of miR-410 on cell viability and cell apoptosis in CRC cells. (A) Transfection efficiency was determined using qRT-PCR. **P<0.05 vs. NC group. (B) Cell Counting Kit-8 assays were performed to evaluate cellular viability in SW-480 and HT-116 cells transfected with miR-410 inhibitor or NC group. (C) Cell apoptosis of SW-480 and HT-116 cells transfected with miR-410 inhibitor or NC was examined by flow cytometry. *P<0.5 vs. NC group. CRC, colorectal cancer; NC, negative control; miR, microRNA.

the DKK1 expression level (Fig. 4C-E). The current results indicate that miR-410 directly targets DKK1 and negatively regulates DKK1 expression.

Effects of miR-410 on Wnt/β-catenin signaling pathways. To further investigate whether miR-410 affected Wnt/β-catenin signaling pathways, SW-480 cells were transfected with miR-410 inhibitor or NC inhibitor. As shown in Fig. 5, western blot analysis revealed that downregulation of miR-410 significantly decreased β-catenin and p-GSK-3β protein levels in SW-480 cells. However, there was no difference in the level of GSK-3β with regard to miR-410 downregulation. Collectively, these results demonstrate that miR-410 may be an important regulator in the Wnt/β-catenin signaling pathways.

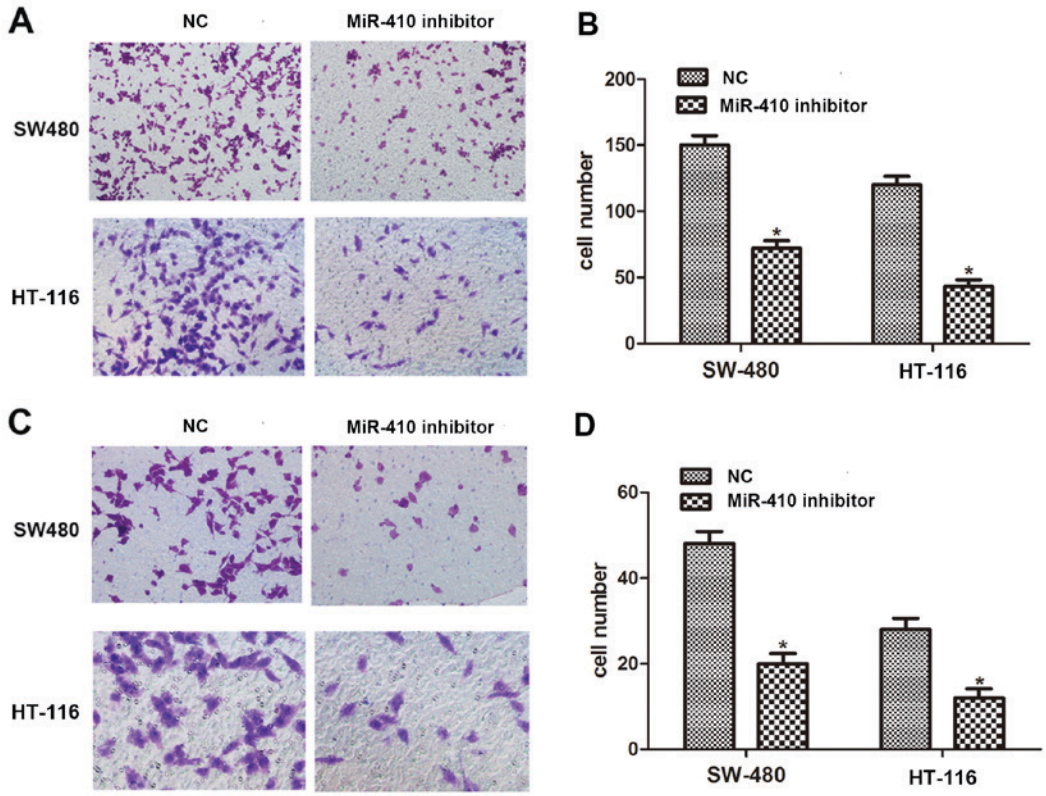

Figure 3. Effects of miR-410 on cell migration and invasion in CRC cells. (A) Migration assay. Representative images of migrated cells on the membrane (magnification, x100). (B) Quantification of migrated cells per field. miR-410 inhibitor inhibited cell migration. (C) Invasion assay. Representative images of invaded cells on the membrane (magnification, x100). (D) Quantification of invaded cells per field. miR-410 inhibitor suppressed cell invasion. *P<0.05 vs. NC inhibitor. CRC, colorectal cancer; NC, negative control; miR, microRNA.

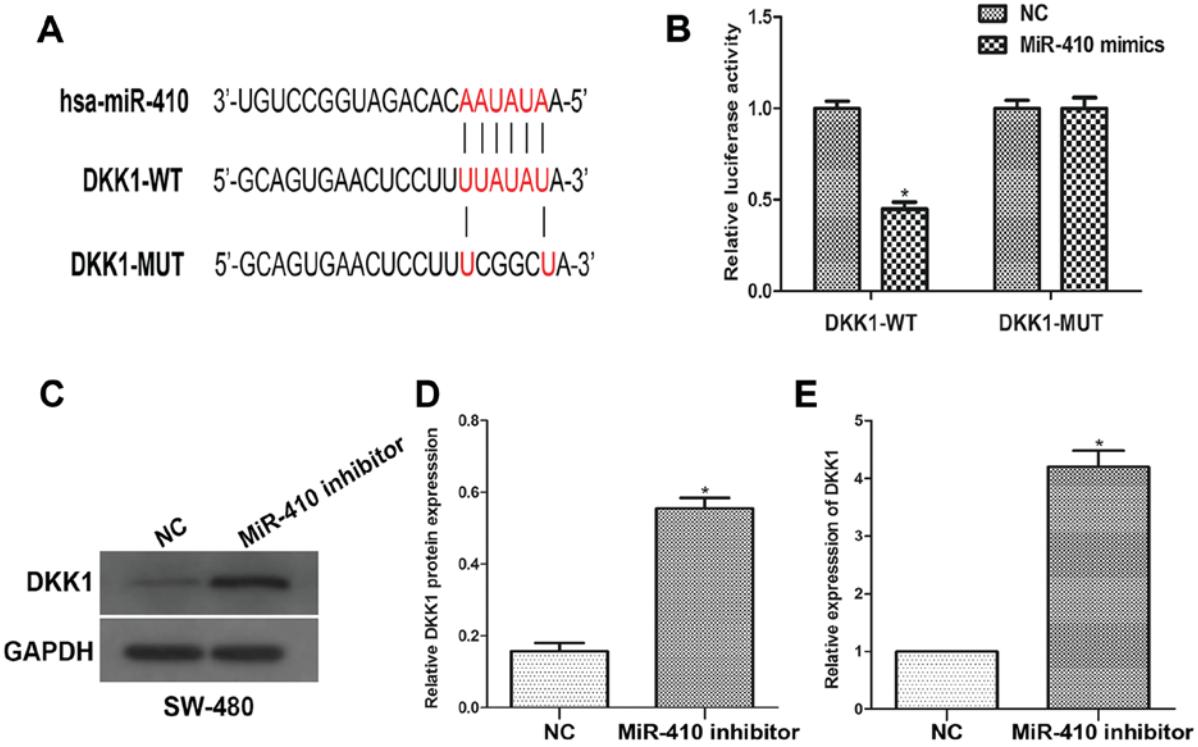

Figure 4. miR-410 directly targets DKK1 and negatively regulates DKK1 expression. (A) Sequence alignment of predicted miR-410 binding sites within the DKK1 3'-UTR and its mutated sequence for luciferase reporter assay. (B) Luciferase reporter assay was performed in SW-480 cells that were co-transfected with miR-410 inhibitor or NC inhibitor and reporter vectors containing DKK1 3'-UTR or mutated DKK1 3'-UTR. Relative luciferase activities are presented. (C-E) Reverse transcription-quantitative polymerase chain reaction and western blot analyses of DKK1 expression after transfection with miR-410 inhibitor in SW-480 cells. *P<0.05 vs. NC inhibitor. DKK1, dickkopf-related protein 1; MUT, mutant; NC, negative control; WT, wild type; miR, micro RNA; 3'-UTR, 3'-untranslated region.

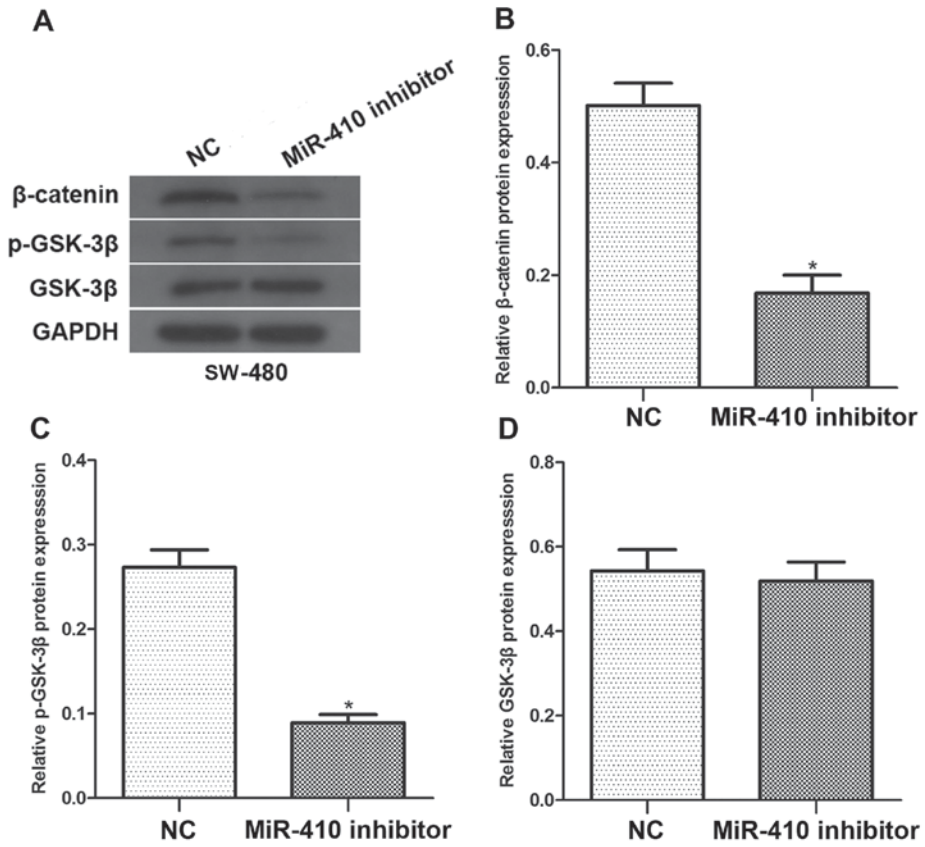

Figure 5. Effects of miR-126 on Wnt/β-catenin signaling pathways. (A) β-catenin, p-GSK-3β and GSK-3β protein levels co-transfected with miR-410 inhibitor or NC in SW-480 cells by western blot analysis. (B) The relative protein level of β-catenin. (C) The relative protein level of p-GSK-3β. (D) The relative protein level of GSK-3β. *P<0.05 vs. NC inhibitor. p-GSK-3β, phosphorylated glycogen synthase kinase-3β; GSK-3β, glycogen synthase kinase-3β; NC, negative control; miR, microRNA.

Discussion

Despite advances in diagnosis and treatment for CRC patients, it remains difficult to eradicate tumors and prevent recurrence. Evidence has demonstrated that 20-45% of patients who undergo radical resection experience relapse or metastasis after a short period of remission (19,20). Therefore, it is important to uncover the underlying molecular mechanisms in the progression of CRC and identify more effective therapeutic targets. Previous studies have revealed that miRNAs closely associate with tumor proliferation and apoptosis in cancer cells by targeting specific genetic targets (6,21). The current study aimed to identify miRNAs that regulate the progression of CRC.

A growing volume of literature has demonstrated that miRNA regulates gene expression post-transcriptionally and acts as an oncogene or tumor suppressor in different cancer types (22,23). miR-410 has been confirmed to inhibit tumor invasion and metastasis in several malignancies (24,25). It has also been reported that miR-410 suppresses CRC cell growth, migration and invasion through binding to certain targets (10,16). In the current study, it was confirmed that endogenous expression of miR-410 was upregulated in CRC cell lines, implying that miR-410 may be involved in the tumorigenesis of CRC. Additionally, the current data demonstrated that miR-410 knockdown inhibited cell proliferation and induced apoptosis in CRC cell lines. Using a cell Transwell assay, it was identified that knockdown of miR-410 mitigated migration and invasion capability of CRC cells *in vitro*. The aforementioned results are in accordance with a previous report (14), suggesting that miR-410 functions like an oncogene in CRC cells and has potential to be used as a biomarker for diagnosis and prognosis of CRC.

The Wnt/β-catenin signaling pathway is well known for its critical role in the early progression of metastasis and tumor growth (26,27). The triggering of Wnt signaling could prevent GSK-3β from activation and maintain β-catenin stabilization, characteristics that are strongly associated with tumor metastasis (28,29). DKK-1 is one of four members of the extracellular Wnt inhibitors family, which can block signaling by binding to plasma membrane Wnt-receptor complexes (30,31). It has been reported that DKK-1 inhibits colorectal cell proliferation both *in vitro* and *in vivo* (32), supporting a tumor suppressor role for this protein. Additionally, several studies have demonstrated that the expression of DKK-1 is downregulated in colorectal adenoma carcinoma at late CRC stages (33). DKK-1 has also been revealed to be downregulated in chronic lymphocytic leukemia and papillary thyroid cancer (34,35). The current study predicted binding between miR-410 and DKK-1 using bioinformatics methods and then confirmed this interaction by luciferase reporter assay. Subsequently, the effect of miR-410 knockdown on the expression of DKK-1 and Wnt/β-catenin signaling was investigated. As the current results revealed, knockdown of miR-410 significantly promoted the expression

of DKK-1 while it decreased the expression of β-catenin and p-GSK-3β. This suggests that the biological function of miR-410 in CRC may be associated with the negative regulation of DKK-1 via the Wnt/β-catenin signaling pathway.

In conclusion, the current study indicated that miR-410 acts as an oncogene in CRC cells by promoting cell proliferation, migration and invasion capacity, and this biological function may, at least in part, be ascribed to the negative regulation of DKK1 through the Wnt/β-catenin signaling pathway. These results implied that miR-410 could potentially be used as a biomarker for the diagnosis and prognosis of CRC.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

WW was responsible for conception and design of the study, data collection and analysis, and manuscript writing. YH designed the study, performed critical revision and supervised all phases of the study. JR and MX, data collection and analysis.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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