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*Original Article*

# Up-regulation of microRNA-19b is associated with metastasis and predicts poor prognosis in patients with colorectal cancer

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**Abstract:** Recent evidence has demonstrated that microRNA-19b (miR-19b) is elevated and functions as a prognosis predictor in hepatocellular carcinoma and melanoma. However, its expression and clinical significance in colorectal cancer (CRC) remain unclear. The study aimed to identify the correlation between miR-19b expression and the clinicopathological features and prognosis of patients with CRC. In this study, we found that the levels of miR-19b were significantly up-regulated in CRC tissues and cell lines compared with matched adjacent non-cancerous tissues and human colon mucosal epithelial cell lines, and its expression was also increased in patients with lymph node metastasis compared with those patients with no lymph node metastasis. Meanwhile, the patients with distal metastasis have a higher miR-19b expression than those patients with no distal metastasis. The high expression of miR-19b in patients with CRC was associated with lymph node metastasis and distant metastasis. miR-19b expression was an independent prognostic indicator for overall survival of CRC patients. Moreover, patients with a high miR-19b expression have shorter overall survival times than those patients with a low miR-19b expression. In addition, an in vitro functional assay showed that miR-19b knockdown restrained the migration and invasion of HCT116 and SW480 cells. In summary, the study provides the first convincing statistical and experimental evidence that the up-regulation of miR-19b is associated with metastasis and predicts unfavorable prognosis in patients with CRC, suggesting that miR-19b may serve as a novel and promising prognostic biomarker in CRC.

**Keywords:** miR-19b, CRC, prognosis, metastasis, biomarker

## Introduction

Colorectal cancer (CRC) is the third most common cancer and one of the most common causes of cancer-related death worldwide [1, 2]. The carcinogenesis of CRC is a multi-step process and malignant transformation with a large number of genetic and epigenetic variations [3]. Although the diagnosis and treatment of CRC have improved, the prognosis of CRC patients is still unsatisfactory [4-6]. Therefore, it is urgent to identify new biomarkers for the treatment of CRC.

MicroRNAs (miRNAs) are a class of single-stranded non-protein-coding RNAs ~22 nt in length which bind to the sequence-complementary of the 3'-untranslated region (3'-UTR) of target genes, resulting in the degradation or translational inhibition of target genes [7, 8]. MiRNAs play important regulatory roles in various pathological and physiological processes, such as differentiation, development, stress response, endocrine homeostasis, inflammation and tumorigenesis [9, 10]. Emerging evidences have demonstrated that the aberrant expression of miRNAs is commonly presented in CRC and associated with tumor initiation and progression [11]. For example, a decreased expression of miR-497 is associated with CRC cancer occurrence, metastasis, advanced stages, and chemoresistance [12, 13]. Up-regulation of miR-155-5p promotes CRC invasion and metastasis [14]. Down-regulation of miR-24-3p is associated with malignant behavior in CRC [15].

**Table 1.** The relationship between miR-19b expression and clinicopathological parameters in patients with CRC

| Parameters | Number of cases | miR-19b expression | | *P* value |
|---|---|---|---|---|
| | | Low (n=17) | High (n=39) | |
| Age (years) | | | | |
| <60 | 25 | 5 | 20 | 0.13 |
| ≥60 | 31 | 12 | 19 | |
| Sex | | | | |
| Male | 37 | 11 | 26 | 0.88 |
| Female | 19 | 6 | 13 | |
| Tumor size | | | | |
| <5 cm | 40 | 12 | 28 | 0.92 |
| ≥5 cm | 16 | 5 | 11 | |
| Differentiation | | | | |
| Well and moderate | 26 | 9 | 17 | 0.51 |
| Poor | 30 | 8 | 22 | |
| Stage | | | | |
| T1-T2 | 22 | 7 | 15 | 0.84 |
| T3-T4 | 34 | 10 | 24 | |
| Location | | | | |
| Rectum | 14 | 4 | 10 | 0.86 |
| Colon | 42 | 13 | 29 | |
| Lymph node metastasis | | | | |
| Absent | 18 | 2 | 16 | 0.03 |
| Present | 38 | 15 | 23 | |
| Distant metastasis | | | | |
| Negative | 43 | 16 | 27 | 0.04 |
| Positive | 13 | 1 | 12 | |

MiR-19b is included in both the miR-106-363 and miR-17-92 clusters, which demonstrates a key oncogenic component of the miRNA cluster in the B-cell transformation model [16]. Previous studies have shown that the upregulation of miR-19b is associated with the poor prognosis of patients with non-small-cell lung carcinoma [17], esophageal squamous cell carcinoma [18], and gastric cancer [19]. In addition, high levels of miR-19b predict a good prognosis in patients with hepatocellular carcinoma [20]. However, the expression and clinical significance of miR-19b in CRC have not yet been well illustrated.

In this study, we investigated the expression pattern of miR-19b in CRC tissues and cell lines. The correlation between miR-19b levels and clinicopathological characteristics was evaluated. Meanwhile, the relationship between miR-19b expression and the prognosis of CRC patients was analyzed. Moreover, the effects of miR-19b on cell invasion and migration in CRC were also explored. Our data demonstrate that the up-regulation of miR-19b is associated with metastasis and predicts adverse prognosis in patients with CRC.

## Materials and methods

### *Clinical sample collection*

A total of fifty-six CRC tissues and matched adjacent non-cancerous tissues (located 2 cm outside of the tumor margin) were collected from patients with CRC in the First Affiliated Hospital of Bengbu Medical College (Bengbu, China) between January 2010 and November 2013. None of the patients had received preoperative treatment. All tissue samples were examined and evaluated by pathologists, according to the 7th edition of the UICC TNM staging system [21]. The tissues were immediately frozen in liquid nitrogen and stored at -80°C until the RNA was isolated. A post-operative follow up was performed every month after discharge or until death. The median follow-up period of patients enrolled in this study was 42.6 months (range 12~77 months). This study was approved by the Ethics Committees of The First Affiliated Hospital of Bengbu Medical College and carried out according to the provisions of the Helsinki Declaration. Written informed consent was obtained from each patient or a legal representative. The detailed clinicopathological data of the patients are shown in **Table 1**.

### *Cell line and cell culture*

The human CRC cell lines (HT29, RKO, HCT116, SW480, and SW620) and the human colon mucosal epithelial cell line (FHC) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA), 100 mg/mL streptomycin sulfate, and 100 U/mL penicillin. The

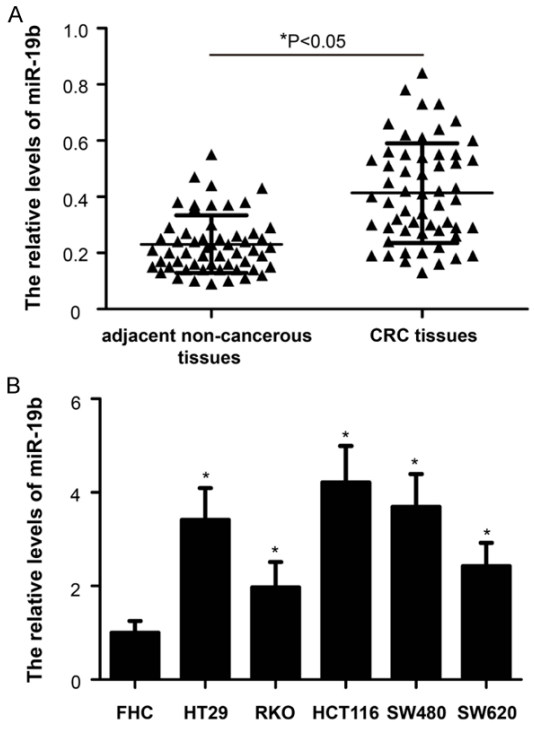


**Figure 1.** Relative expression levels of miR-19b in CRC tissues and cell lines. A. miR-19b expression had a higher expression in CRC tissues than in the matched adjacent non-cancerous tissues. B. A qRT-PCR assay showed that miR-19b expression was up-regulated in CRC cell lines (HT29, RKO, HCT116, SW480, and SW620) compared with the human colon mucosal epithelial cell line (FHC). CRC: colorectal cancer. *P<0.05.

cells were incubated in a humidified atmosphere containing 5% $CO_2$ at 37°C.

*Total RNA isolation*

Total RNA was extracted from CRC tissues and cell lines using TRIzol reagent (Invitrogen,CA, USA), according to the manufacturer protocols. RNA concentration was quantitated using a Thermo Scientific NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, DE, USA). Only those RNA samples with 260/280 ratios at 1.8~2.0 were used for further experiments.

*Quantitative reverse transcription PCR (qRT-PCR)*

The first strand of cDNA (complementary DNA) was synthesized using a miScript Reverse Transcription Kit (Qiagen, Hilden, Germany) at a final volume of 20 μl, including 6 μl RNA, 4 μl 5 × miScript RT buffer, and 2 μl miScript Reverse Transcriptase Mix and 8 μl RNase-free water. The reaction was performed at 37°C for 60 min, at 95°C for 5 min, and then stored at 4°C. The qRT-PCR assay was conducted using a miScript SYBR® Green PCR Kit (Qiagen, Hilden, Germany) and the Roche LightCycler 480 Real-Time PCR System. The following primers were used: miR-19b sense primer, 5'-TGTGCAAATCCATGCAAAACTGA-3'; U6 small nuclear RNA (U6) sense primer, 5'-CTCGCTTCGGCAGCACA-3'; both of the antisense primers were provided by the miScript SYBR® Green PCR Kit. The reaction was incubated in a 20 μl final volume and was done for 40 cycles (20 s at 95°C, 30 s at 55°C, and 30 s at 72°C). The expression levels of miR-19b were normalized to U6 and were determined by using the delta-delta Ct method [22].

*Cell invasion assay*

Cell invasion was determined by Transwell assays. The miR-19b antagomir (antagomir) or antagomir negative control (antagomir control) was transfected into SW480 cells according to the manufacturer's protocol. Subsequent to incubation for 24 h at 37°C, about $6 \times 10^4$ cells were transferred to the top of the Matrigel-coated invasion chambers (BD Biosciences, CA, USA) in a serum-free DMEM. DMEM containing 10% FBS was added to the lower chamber. 48 h after transfection, the invading cells were fixed using 95% ethanol and stained with 0.1% crystal violet at 37°C. A Leica light microscope (DM4000B; Leica Microsystems GmbH, Wetzlar, Germany) was used to observe invasive cells.

*Cell migration assay*

A migration scratch assay was used to assess the migratory ability of the CRC cell lines. HCT116 cells were seeded into 6-well plates and transfected with antagomir or antagomir control. Following 6 h of transfection, confluent monolayer cells were then scraped with a 10 μl pipette tip to generate scratch wounds and washed twice with media to remove cell debris. Time lapse images were captured after 12 h. Image was captured by a Leica light DM4000B microscope from five randomly selected fields in each sample.

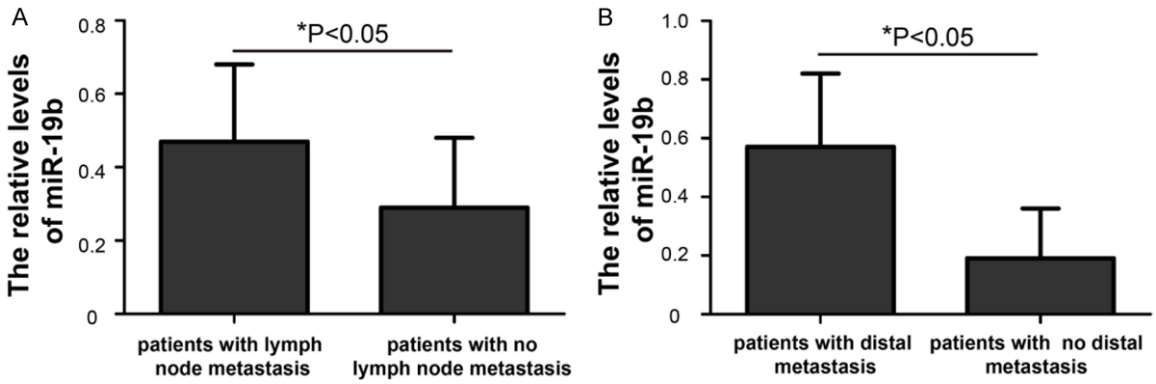


**Figure 2.** Up-regulation of miR-19b is associated with metastasis in patients with CRC. A. Expression levels of miR-19b were detected in patients with lymph node metastasis or no lymph node metastasis. B. qRT-PCR assay revealed that patients with distal metastasis have higher miR-19b expression than those patients with no distal metastasis. *P<0.05.

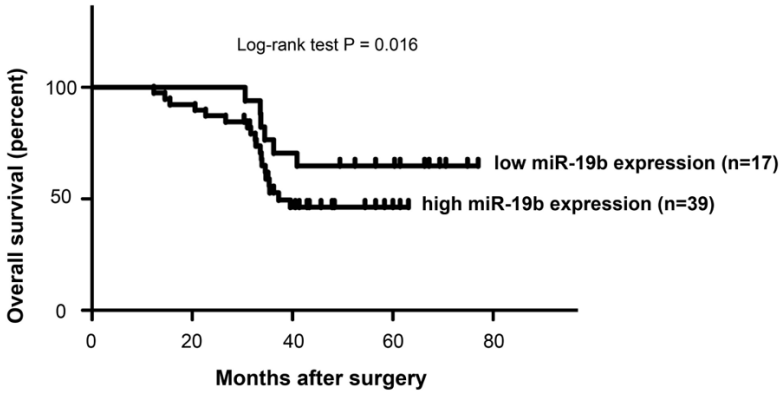


**Figure 3.** Kaplan-Meier survival curves of patients with CRC based on miR-19b expression status. Patients in high miR-19b expression group had shorter survival times than those patients in low miR-19b expression group. *P<0.05.

*Statistical analysis*

Statistical analysis was performed using SPSS v19.0 software (SPSS, Inc., Chicago, IL, USA). The expression levels of miR-19b in CRC tissues and adjacent non-cancerous tissues were analyzed using Student's t test. One-way ANOVA was used to assess miR-19b expression in CRC and FHC cell lines. A chi-square test was performed to determine the relationship between miR-19b levels and clinicopathological features. Kaplan-Meier and log-rank testing was applied to evaluate the effect of miR-19b on the overall survival of patients with CRC. The significance of survival variables was evaluated using univariate and multivariate Cox proportional hazards regression analyzes. A value of P<0.05 were considered statistically significant.

## Results

*Increased expression of miR-19b in CRC tissues and cell lines*

To determine the expression levels of miR-19b in CRC, a qRT-PCR assay was performed to measure its expression in 56 pairs of CRC tissues and adjacent non-cancerous tissues. As shown in **Figure 1A**, the levels of miR-19b in the CRC tissues were significantly up-regulated compared to the matched adjacent non-cancerous tissues (P<0.05). We also examined miR-19b expression in five human colorectal cancer cell lines (HT29, RKO, HCT116, SW480, and SW620) and a human colon mucosal epithelial cell line (FHC) by qRT-PCR. The results showed that miR-19b expression was clearly higher in five CRC cell lines relative to FHC (**Figure 1B**, P<0.05).

*Up-regulation of miR-19b is associated with metastasis in patients with CRC*

A qRT-PCR assay was applied to explore miR-19b expression in lymph node metastasis and distal metastasis of patients with CRC. The results revealed that miR-19b levels were increased in patients with lymph node metastasis compared with those patients with no lymph node metastasis (**Figure 2A**, P<0.05). Mean-

**Table 2.** Univariate Cox regression analysis of prognostic parameters in patients with CRC

| Parameters | Univariate Cox regression | |
|---|---|---|
| | HR (95% CI) | *P* value |
| Age (<60 vs. ≥60) | 0.83 (0.31-2.06) | 0.24 |
| Sex (Male vs. Female) | 1.12 (0.42-2.77) | 0.17 |
| Tumor size (<5 cm vs. ≥5 cm) | 1.57 (0.64-3.59) | 0.55 |
| Differentiation (Well and moderate vs. Poor) | 2.09 (0.71-4.76) | 0.43 |
| Stage (T1-T2 vs. T3-T4) | 1.44 (0.50-2.93) | 0.19 |
| Location (Rectum vs. Colon) | 1.84 (0.68-4.13) | 0.38 |
| Lymph node metastasis (Absent vs. Present) | 2.73 (1.04-9.32) | <0.01 |
| Distant metastasis (Negative vs. Positive) | 1.62 (0.47-7.73) | <0.01 |
| miR-19b (Low vs. High) | 3.16 (1.19-11.96) | <0.01 |

**Table 3.** Multivariate Cox regression analysis of prognostic parameters in patients with CRC

| Parameters | Multivariate Cox regression | |
|---|---|---|
| | HR (95% CI) | *P* value |
| Age (<60 vs. ≥60) | - | - |
| Sex (Male vs. Female) | - | - |
| Tumor size (<5 cm vs. ≥5 cm) | - | - |
| Differentiation (Well and moderate vs. Poor) | - | - |
| Stage (T1-T2 vs. T3-T4) | - | - |
| Location (Rectum vs. Colon) | - | - |
| Lymph node metastasis (Absent vs. Present) | 2.06 (0.90-6.43) | 0.28 |
| Distant metastasis (Negative vs. Positive) | 1.31 (0.38-6.05) | 0.16 |
| miR-19b (Low vs. High) | 3.47 (1.61-13.70) | 0.03 |

while, patients with distal metastasis have higher miR-19b levels than those patients with no distal metastasis (**Figure 2B**, P<0.05). Collectively, these data suggest that the up-regulation of miR-19b is associated with metastasis in CRC

*Association of miR-19b expression with clinicopathological characteristics in patients with CRC*

We further evaluated the relationship of miR-19b expression with clinicopathological parameters. Patients were categorized into a high miR-19b expression group and a low miR-19b expression group based on the median level of miR-19b. Of the 56 CRC patients, 17 fell in the low miR-19b expression group and 39 fell in the high miR-19b expression group. As shown in **Table 1**, a positive correlation was observed in the high miR-19b expression group with lymph node metastasis and distant metastasis (P<0.05). There was no significant association between miR-19b expression and other clinicopathological features, such as age, sex, tumor size, location, stage, or differentiation. These results suggest that an elevated expression of miR-19b is associated with CRC progression.

*Correlation of miR-19b expression with prognosis in patients with CRC*

Among the 56 CRC patients, 25 (44.6%) died as a result of cancer progression during the follow-up period. CRC patients with high miR-19b levels had significantly shorter survival times compared with those with low miR-19b expression levels (**Figure 3**, P<0.05). As shown in **Table 2**, in univariate analysis, lymph node metastasis, distant metastasis, and miR-19b expression were associated with poor overall survival in patients with CRC. In multivariate analysis, only miR-19b expression was an independent prognostic factor for overall survival of CRC patients (**Table 3**, P<0.05).

*miR-19b knockdown inhibited cell migration and invasion in CRC*

To explore the functional roles of miR-19b in CRC cells, antagomir or an antagomir control was transfected into HCT116 and SW480 cells. The transfection efficiency was analyzed by fluorescence microscopy 6 h after transfection. As shown in **Figure 4A**, the transfection efficiency was ~56.3 and 44.7% in HCT116 and SW480 cells, respectively. The fold changes to miR-19b expression after treatment with antagomir, as determined by a qRT-PCR assay, were 0.35 and 0.26 in the HCT116 and SW480 cells compared to cells treated with the antagomir control, respectively (**Figure 4B**, P<0.05).

Images of the scratches were captured in HCT116 cells at 0 and 12 h after transfection

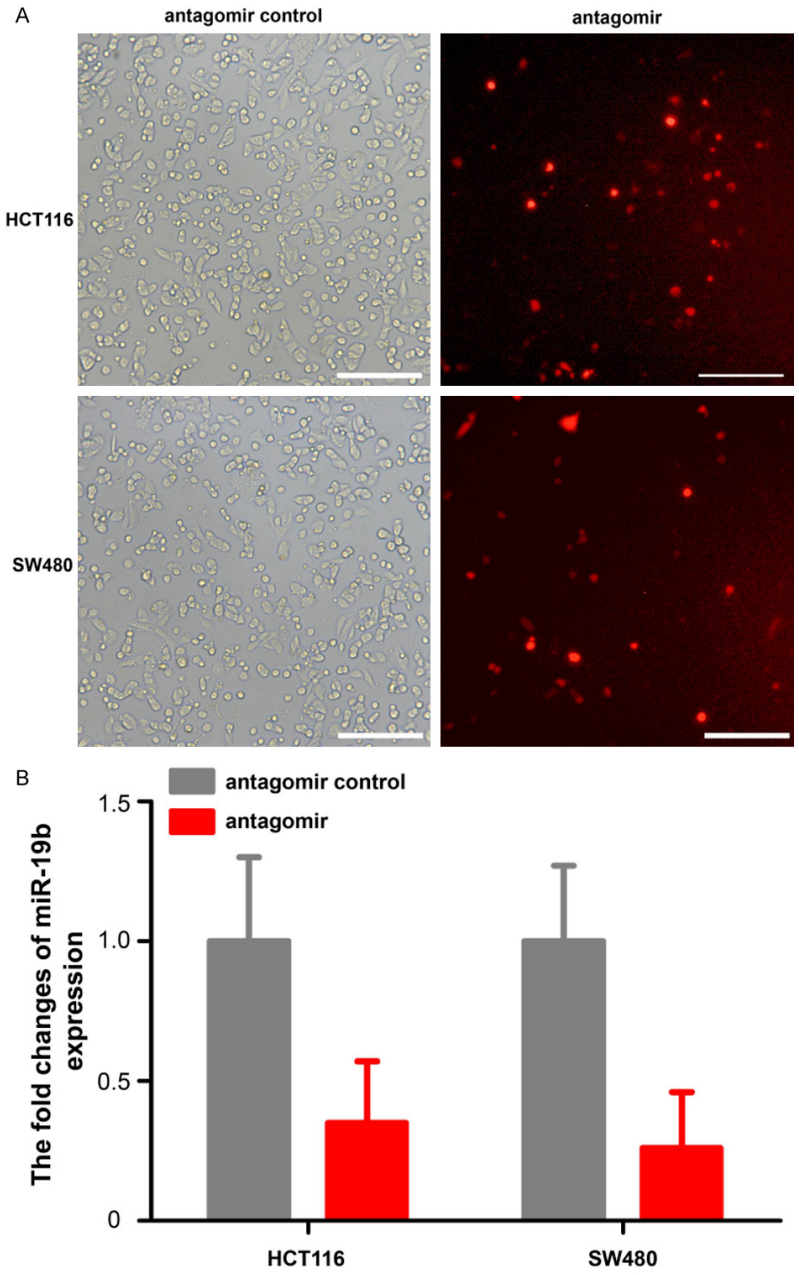


**Figure 4.** Analysis of transfection efficiency and miR-19b levels by fluorescence microscopy and qRT-PCR assay. A. Images of HCT116 and SW480 cells transfected with miR-19b antagomir (antagomir) or antagomir negative control (antagomir control). Bar = 200 μm. B. The fold changes of miR-19b expression in HCT116 and SW480 cells treated with antagomir or the antagomir control were analyzed 48 h after transfection. *P<0.05.

(**Figure 5A**). The results demonstrated that the migratory distances of the HCT116 cells transfected with antagomir were markedly shorter, as compared with the antagomir control group (P<0.05). Transwell assay indicated that the knockdown of miR-19b significantly reduced the invasion of the SW480 cells compared to the antagomir control group (**Figure 5B**, P<0.05). These results indicate that the reduction of miR-19b expression suppresses the migration and invasion of RCC cells.

## Discussion

CRC has been described as a multistep malignancy due to the progressive accumulation of changes involving key oncogenes or tumor suppressors [3]. Mounting studies have demonstrated that miRNAs are aberrantly expressed in CRC tissues and exhibit a tumor suppressive or oncogenic role in the development and progression of CRC [23, 24]. A better understanding of the changes of miRNAs in CRC may facilitate a better acknowledgement of the mechanisms of carcinogenesis and make it possible to improve the diagnosis and therapy of CRC.

In the literature, miR-19b has been shown to play an important role in thrombosis and the aging process, as well as in cardiovascular diseases [25-28]. The aberrant expression of miR-19b is a common event in various human cancers, suggesting that miR-19b plays a key role in tumorigenesis [29-31]. Nonetheless, the expression and clinical significance of miR-19b in CRC still needs to be further illustrated. In this study, we analyzed the expression of miR-19b in CRC tissues and cell lines. Our data showed that miR-19b levels were significantly elevated in CRC tissues and cell lines compared with matched adjacent non-cancerous tissues and human colon mucosal epithelial cell line, which agreed with findings from previous studies [29-31].

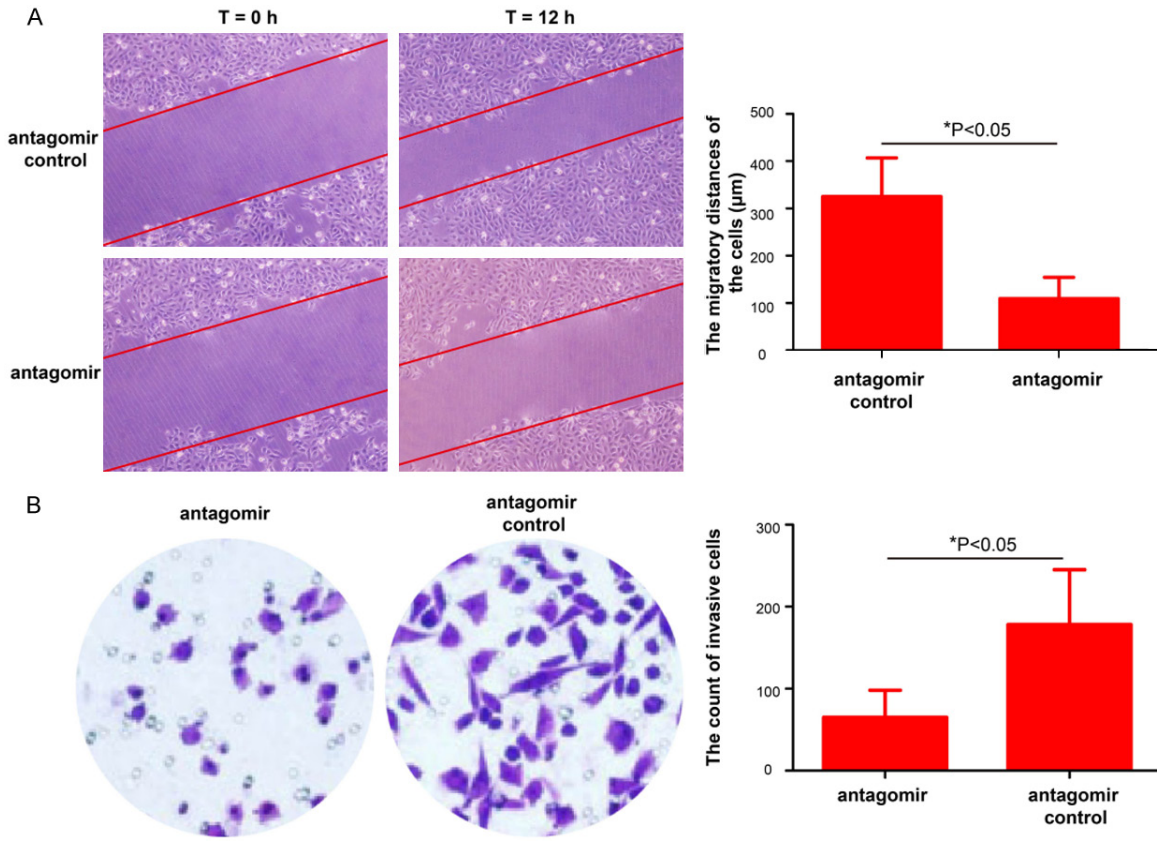


**Figure 5.** miR-19b knockdown inhibited cell migration and invasion in CRC. A. Images of the HCT116 cells transfected with antagomir or antagomir control at 0 h and 12 h after the scratches were made at the same point. The lines indicated the migratory front. B. Transwell assay analysis of invasion of SW480 cells transfected with antagomir or antagomir control. *P<0.05.

We then investigated the correlation between miR-19b expression and the clinicopathological features of patients with CRC. The results showed that a high expression of miR-19b in patients with CRC was associated with lymph node metastasis and distant metastasis. Metastasis is an important factor in the development of CRC [15]. Here, we also found that patients with lymph node or distal metastasis had a higher miR-19b expression than those patients with no lymph node or distal metastasis. Further studies showed that miR-19b knockdown could suppress CRC cell migration and invasion in vitro, suggesting that a high expression of miR-19b is associated with metastasis of CRC.

To further determine the potential prognostic role of miR-19b, we investigated the association between miR-19b levels and overall survival in patients with CRC. A Kaplan-Meier survival analysis revealed that patients with high miR-19b expression had poorer survival times compared with those patients with low miR-19b expression. Moreover, univariate and multivariate Cox regression analyzes indicated that high expression of miR-19b is an independent prognostic factor for CRC patients. Similar results from other miRNAs have been reported in relation to CRC. For instance, Liu et al. [32] reported that up-regulation of miR-1260b is associated with poor prognosis in patients with CRC. Zheng et al. demonstrated that down-regulation of miR-422a is an independent prognostic factor in CRC [33]. Tang et al. showed that a decreased expression of miR-452 is an independent factor predicting poor prognosis for CRC patients [34]. Those findings suggest that miRNAs may be related to cancer patients' clinicopathological characteristics and prognosis. As a result, miR-19b is a novel promising biomarker for the prognosis of CRC.

In summary, our results provide the first evidence that miR-19b is up-regulated in CRC tissues and cell lines. Up-regulation of miR-19b is

associated with metastasis and predicts unfavorable prognosis in patients with CRC. Further studies are still required to explore the molecular mechanism of miR-19b in CRC.

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## Disclosure of conflict of interest

None.

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