



The miRNA125a-5p and miRNA125b-1-5p cluster induces cell invasion by down-regulating DDB2-reduced epithelial-to-mesenchymal transition (EMT) in colorectal cancer

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Background: MicroRNA (miRNA) is a kind of non-coding RNA that regulates gene expression and is involved in tumor development. MiRNA-125 is reportedly aberrantly expressed in colorectal cancer tissue; however, its potential function and underlying mechanism remain unclear. The present study aimed to investigate the expression level and potential role of the miRNA-125 family in the invasion and migration of colorectal cancer.

Methods: To further understand the role of the miRNA-125 family in metastatic colorectal cancer, we overexpressed miRNA-125 in the SW480 cell line by transfection with the miRNA-125 family mimics or a sponge. Methyl thiazolyl tetrazolium (MTT) assay was performed to identify the effect of the miRNA-125 family on cell proliferation, and a Transwell filter assay was used to detect the role of the miRNA-125 family in migration and invasion. A luciferase assay was carried out to confirm the binding site of miRNA-125 and the target gene, damage specific DNA binding protein 2 (*DDB2*). Western blot was applied to detect the expression levels of *DDB2* and the markers of epithelial-to-mesenchymal transition (EMT) in colorectal cancer cells.

Results: The real-time polymerase chain reaction (PCR) results showed that miR-125a-5p and miR-125b-1-5p were up-regulated in metastatic colorectal cancer tissues. The Transwell filter assay results appeared that miR-125a-5p and miR-125b-1-5p could promote the invasion and migration of colorectal cancer cells. The luciferase assay data confirmed the binding site of miR-125a-5p and miR-125b-1-5p on the 3' untranslated region (3'UTR) of *DDB2* messenger RNA (mRNA). The real-time PCR and Western blot results indicated that miR-125a-5p and miR-125b-1-5p could regulate the expression levels of *DDB2* and EMT markers, and lower *DDB2* expression was observed in metastatic tissues.

Conclusions: Our findings illustrated that miRNA125a-5p and miRNA125b-1-5p could reduce the expression of *DDB2* by binding to the 3'UTR region, and then regulate the expression levels of EMT markers, leading to the enhanced invasion and metastasis of colorectal cancer cells. Thus, miRNA125a-5p and miRNA125b-1-5p might be novel markers of colorectal cancer migration and potential therapeutic targets to treat metastatic colorectal cancer patients.

Keywords: Colorectal cancer (CRC); metastases; miRNA-125; damage specific DNA binding protein 2 (DDB2); endothelial-to-mesenchymal transition

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive system, and its increasing rates of morbidity and mortality have attracted greater attention in recent decades (1). Approximately 20 percent of CRC cases have reportedly already metastasized at the time of diagnosis, and the 5-year survival rate of metastatic CRC from 1975 to 2009 is only 5.5–12.5% (2). The incidence rate of CRC has increased by 22% from 2000 to 2013 among adults below the age of 50 years. Similarly, the mortality rates increased by 13% in patients under the age of 50 years between 2000 and 2014 (3). However, the invasion and metastasis mechanisms of CRC are poorly understood, which presents a significant problem for clinicians.

MicroRNA (miRNA) is an endogenous small non-coding RNA with a length of about 22 nucleotides, which play crucial roles in important biological processes by regulating multiple proteins expression. Acting at the post-transcriptional level (4–6), miRNA can modulate the expression of as much as 30% of all mammalian protein-encoding genes (7). In some cases, miRNAs are encoded by

multiple loci, some of which are organized in tandemly co-transcribed clusters. With the in-depth research on miRNA in recent years, an increasing number of miRNAs have been found to play a key regulatory role in human cancers (8–10), including breast (11), bladder (12), lung (13), and gastric (14) cancers.

MiR-125 is a highly conserved miRNA transcribed from three different clusters in humans, namely, miR-125a on chromosome 19, miR-125b-1 on chromosome 11, and miR-125b-2 on chromosome 21. MiR-125b-1 and miR-125b-2 code for the same mature sequence, and their miRNAs (located in the introns of protein-coding genes) contain putative promoters that regulate transcription independently of their host gene, which differs from most miRNA mechanisms. In addition to paralogs that code for the same mature sequence, there are homologs (e.g., miR-125a) that code for different mature sequences, which contain the same seed region, and therefore, might have similar functions (15). According to previous investigations that employed a next-generation sequencing (NGS)-based profiling study of miRNAs (miRNome), miRNA-125 is present in metastatic CRC, which indicates that there is a close relationship between miR-125 and metastatic CRC (16,17). Additionally, miRNA-125 is positively correlated with hematopoietic malignancy (18). Based on the above findings, we speculate that miRNA-125 might participate in the regulation of CRC metastasis.

In recent decades, numerous studies have demonstrated that the invasion of cancer cells could be induced by several physicochemical factors (19–21); however, their relationship with tumor metastasis is still unknown. Tumor metastasis is a key process of cancer development and occurs via a series of steps, and is the main reason for treatment failure (22). Most CRC patients are only found after metastasis, and the prognosis is poor. Before invasion, cancer cells need to pass through the epithelial-to-mesenchymal transition (EMT) process to obtain migration and invasive properties (23). The essential steps in this process include the loss of cell polarity and achieving a mesenchymal phenotype, which are accompanied by decreased expression of the

Highlight box

Key findings

- The miRNA125a-5p and miRNA125b-1-5p could reduce the expression of DDB2 by binding to the 3'UTR region, and then regulate the expression levels of EMT markers, leading to the enhanced invasion and metastasis of colorectal cancer cells.

What is known and what is new?

- MiRNA-125 is reportedly aberrantly expressed in colorectal cancer tissue;
- The present study explained the mechanism of miRNA-125 family regulated invasion and migration of colorectal cancer.

What is the implication, and what should change now?

- The miRNA125a-5p and miRNA125b-1-5p might be novel markers of colorectal cancer migration and potential therapeutic targets to treat metastatic colorectal cancer patients.

epithelial marker, E-cadherin, and increased expression of mesenchymal markers, such as N-cadherin and Vimentin (24). However, the potential function and underlying metastasis mechanism of CRC remain unclear, and thus, further investigation is needed (25).

In this study, we aimed to reveal the potential function and mechanism of the miR-125 family in CRC cell lines. We found that miR-125a-5p and miR-125b-1-5p could not only reduce the expression of their targeted gene [damage specific DNA binding protein 2 (DDB2)] but also inhibited the expression of epithelial markers and increased those of mesenchymal cell markers in cancer cells, which was mediated by DDB2. This study might provide helpful information in understanding the function and mechanism of miR-125 family-regulated CRC migration. We present the following article in accordance with the MDAR reporting checklist (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-1222/rc>).

Methods

Patients and samples

Patients were identified using the electronic records obtained from The First Affiliated Hospital of Nanjing Medical University. Samples were collected from 60 CRC patients from 2012 to 2015, including primary tumors (n=30) and paired metastatic niches (n=30). The methods were performed in accordance with the approved guidelines by the Ethics Committee of The First Affiliated Hospital of Nanjing Medical University (No. 2010-SR-091). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from all subjects.

Cell lines and cell culture

The SW480 human CRC cell line was obtained from the American Type Culture Collection (ATCC, CCL-228, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 50 mg/mL penicillin (Gibco), 50 mg/mL streptomycin (Gibco), and 1 mM sodium pyruvate. The cells were then cultured at humidified 37 °C in 5% carbon dioxide (CO₂) and the cell culture medium was replaced every 48 hours.

Transient transfection

Before transfection, SW480 cells were seeded into six-well plates and incubated until 60–70% confluence. The cells were then transfected with miR-125a-5p mimics/sponge, miR-125b-1-5p mimics/sponge, miR-125a-5p and miR-125b-1-5p mimics, miR-125a-5p and miR-125b-1-5p sponge, respectively. Lipo2000 was used for transfection according to the manufacturer's instructions.

Methyl thiazolyl tetrazolium assay

The SW480 cells were seeded into 96-well plates at a density of 4,000 cells per well and incubated overnight. Forty-eight hours after transfection, the cells were then treated with 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma Aldrich, USA) solution [5 mg/mL in phosphate-buffered saline (PBS)] and incubated for 4 h at 37 °C. Next, the medium was rapidly removed and the MTT crystals were solubilized in 100 µL dimethyl sulfoxide (DMSO). The optical density (OD) volume was determined at 490 nm for each well using a microplate spectrophotometer (BioTek, USA). The data represented the mean ± standard deviation (SD) of at least three independent experiments.

Cell invasion assay

Briefly, the upper surface of the Transwell filter (8.0 µm in pore size) was coated with 40 µg extracellular matrix (ECM) gel before being air-dried overnight at room temperature. Approximately 4×10⁴ cells were added into the upper chamber and cultured overnight. The cells were then transfected with the miRNA mimics/sponge for 6 h, respectively. After 72 h of incubation at 5% CO₂ and 37 °C, the cells invaded the opposite surface of the filter. The filters were fixed with 4% paraformaldehyde and stained with crystal violet, and the cells on the upper surface were then removed completely with a cotton swab. Cell migration was observed under light microscopy (ZEISS, German) at a magnification of ×100. The cells were then lysed using an extraction buffer (Millipore, ECM210, USA) and collected for the detection of OD at 570 nm; the OD value of the treated cells was normalized to the control. Tumor cell invasiveness was defined as the average OD of the cells.

Luciferase reporter assays

The SW480 cells were seeded into 48-well plates at a density of 10,000 cells per well and incubated overnight, then co-transfected with pTA-8×Luc, pRL-TK, and synthetic *DDB2* including wild type (WT) or mutant type (MUT). After 48 h, the cells were rinsed with PBS and lysed using a 100 µL passive lysis buffer (PLB). The luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega, USA) and a luminometer. The luciferase activity of each lysate was normalized to the activity of Renilla luciferase driven by the constitutively expressing promoter in the pRL-TK vector.

RNA extraction and quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using Trizol (TaKaRa, Japan) reagent according to the manufacturer's protocol. Total RNA concentrations were determined using a biophotometer (Eppendorf, Germany). Then, 1 µg of purified RNA was reverse transcribed to complementary DNA (cDNA) using the PrimeScript RT Reagent Kit (TaKaRa, Japan). For quantitative real time PCR (qRT-PCR), each reaction was conducted in 96-well plates, with a final volume of 20 µL, including 10 µL SYBR Green PCR Master Mix (TaKaRa, Japan) plus 1 µL primers (2 µM) (Table S1), 1 µL of template DNA, and 7 µL H₂O. Each reaction was run in triplicate. Gene expression levels were determined using the comparative threshold cycle $2^{-\Delta\Delta C_t}$ analysis method.

Western blotting

After collection of the cell protein components, a standard Western blotting analysis was employed to investigate the protein expression levels. Briefly, the protein extracts were prepared at the indicated time point using KEYGEN Protein Extraction Kit (KEYGEN, China). Protein lysates were boiled in sodium dodecyl sulfate (SDS) sample loading buffer for 5 minutes, then subjected to 10% SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA). The membranes were blocked for 1 hour in 5% milk-Tris-Buffered Saline Tween-20 (TBST) at room temperature and incubated overnight at 4 °C with anti-DDB2 (Abcam, ab51017, UK) and anti-GAPDH (Proteintech, 60004-1, China) antibodies, respectively.

The membranes were washed three times with TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Proteintech, SA00001-1, China) at room temperature for 2 h. The blots were visualized using enhanced chemiluminescence (Thermo, USA) and analyzed using a scanning densitometer with the molecular analysis software FluorChem M system (Protein Simple, USA).

Statistical analysis

The differences in the invasion rate and protein expression levels between the treated cells and the controls were assessed by one-way analysis of variance (ANOVA) method followed by the standard student *t*-test analysis. A P value of less than 0.05 was considered statistically significant (P value <0.05). Each experiment was performed at least three times. Computer-based calculations were conducted using SPSS version 20 (SPSS Inc., Chicago, Illinois, USA).

Results

MiRNA-125 family expression characters in primary colorectal tumors and paired metastatic niches

To further explore the relationship between the miRNA-125 family (miR-125a-5p, miR-125b-1-5p, and miR-125b-2-5p) with primary and metastatic tumors, the expression levels of the miRNA-125 family were determined by RT-PCR. The results revealed that miR-125a-5p (Figure 1A) and miR-125b-1-5p (Figure 1B), and not miR-125b-2-5p (Figure 1C), were highly expressed in metastatic tumors compared to primary tumors, indicating that miR-125a-5p and miR-125b-1-5p are correlated with CRC metastasis.

Overexpression of miR-125a-5p and miR-125b-1-5p increases cell invasiveness

A Transwell-filter assay was performed to identify the roles of miR-125a-5p and miR-125b-1-5p in CRC cell invasion. SW480 cells were seeded in a 24-well ECM-coated Transwell chamber and transfected with miR-125a-5p mimics/sponge, miR-125b-1-5p mimics/sponge, miR-125a-5p and miR-125b-1-5p mimics/sponge, respectively. The invaded cells were stained with crystal violet and statistically analyzed by the OD volume.

Compared to the mimics control group (Figure 2A, 1), the miR-125a-5p mimics (Figure 2A, 2) and miR-125b-1-5p mimics (Figure 2A, 3) increased cell invasion ability,

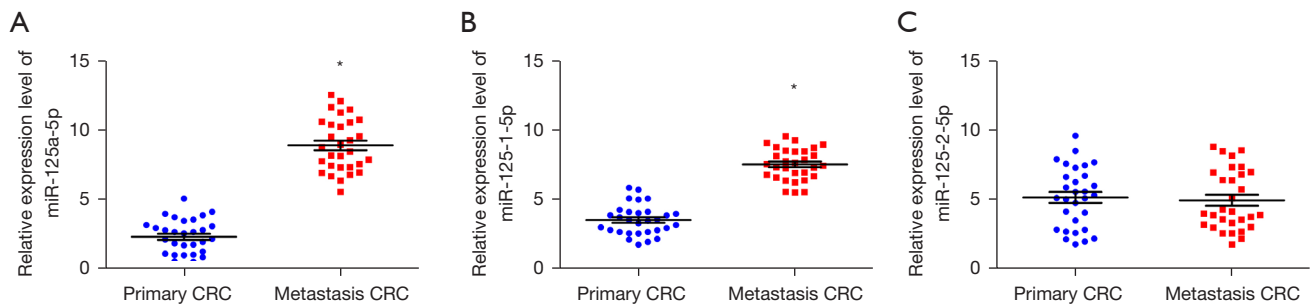


Figure 1 Expression levels of the miR-125 family in primary and metastatic colorectal tumor tissues. (A) MiR-125a-5p was highly expressed in metastatic niches. (B) MiR-125b-1-5p was significantly up-regulated in metastatic niches. (C) There was no notable difference in miR-125b-2-5p expression between primary and metastatic tissues. *, $P < 0.05$. CRC, colorectal cancer.

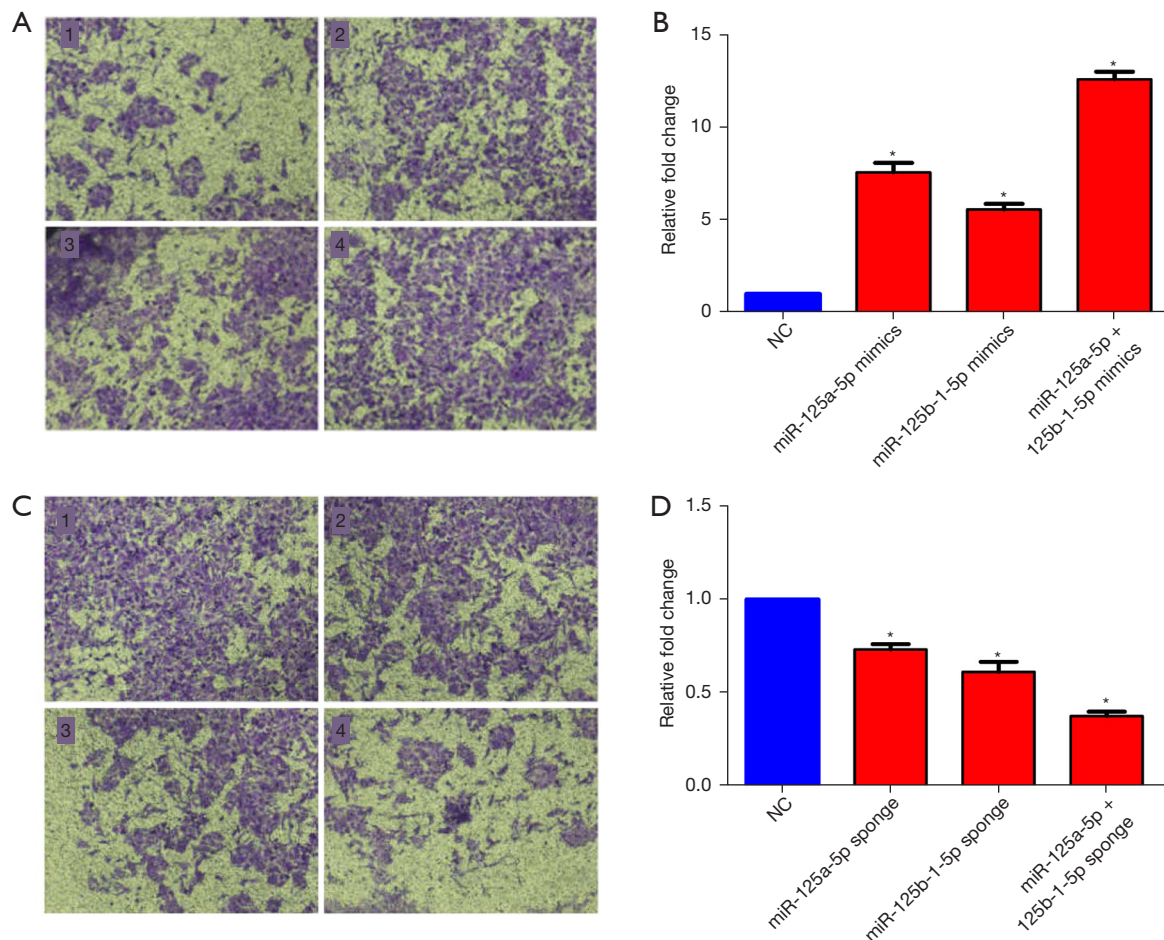


Figure 2 Overexpression of miR-125a-5p and miR-125b-1-5p increases cell invasiveness. (A) Compared to the mimics control group [1], miR-125a-5p [2] and miR-125b-1-5p [3] respectively increased the cell invasion ability, and miR-125a-5p combined with miR-125b-1-5p [4] enhanced the cell invasion ability with a synergistic effect (crystal violet staining, 100 \times). (B) The invaded cells were statistically analyzed. (C) Meanwhile, compared to the sponge inhibitor control group [1], the miR-125a-5p sponge [2] and the miR-125b-1-5p sponge [3] respectively reduced the cell invasion ability, and the miR-125a-5p sponge combined with the miR-125b-1-5p sponge [4] reduced the cell invasion ability with a synergistic effect (crystal violet staining, 100 \times). (D) The invaded cells were statistically analyzed. *, $P < 0.05$. NC, negative control.

respectively, and the miR-125a-5p mimics combined with the miR-125b-1-5p mimics enhanced cell invasion ability, exhibiting a synergistic effect (Figure 2A, 4). The statistically analyzed results revealed that the miR-125a-5p mimics and miR-125b-1-5p mimics increased cell invasiveness by 7.56 and 5.56 folds, respectively, and the combination of miR-125a-5p mimics/miR-125b-1-5p mimics increased cell invasiveness by 12.59 folds (Figure 2B).

Meanwhile, compared to the sponge inhibitor control group (Figure 2C, 1), the miR-125a-5p sponge (Figure 2C, 2) and miR-125b-1-5p sponge (Figure 2C, 3) reduced the cell invasion ability, respectively, and the miR-125a-5p sponge combined with the miR-125b-1-5p sponge also reduced the cell invasion ability with a synergistic effect (Figure 2C, 4). The statistically analyzed results revealed that the miR-125a-5p sponge and miR-125b-1-5p sponge reduced cell invasiveness by 26.12% and 32.88%, respectively, and the combination of the miR-125a-5p sponge /miR-125b-1-5p sponge reduced cell invasiveness by 46.29% (Figure 2D).

MiRNA-125a-5p and miR-125b-1-5p inhibit DDB2 expression by binding to 3'UTR regions of the DDB2 mRNA

To investigate the potential mechanism of miRNA-125 in regulating colorectal invasion, we primarily predicted *DDB2* as the miRNA-125 downstream via crossover analysis in the miRBase, miRDB, and TargetScan databases (Figure 3A). The results showed miR-125a-5p and miR-125b-1-5p bind to the 3'UTR region of *DDB2* at positions 221–228 through the “GAGUCCCU” motif.

We applied a Dual-Luciferase Reporter Assay to verify the binding sites. We constructed the WT and MUT (CAGG mutated to ACUU) luciferase reporter plasmids of the *DDB2* 3'UTR region (Figure 3A), then and co-transfected these with miR-125a-5p and miR-125b-1-5p into SW480 cells, respectively. The results showed that compared to the MUT-*DDB2* groups, both miR-125a-5p and miR-125b-1-5p could reduce the expression of WT *DDB2* with a synergistic effect (Figure 3B), which is consistent with the invasion assay.

We then explored the influence of miR-125a-5p and miR-125b-1-5p on *DDB2* expression levels in-depth. A *DDB2* expression vector plasmid was constructed for RT-PCR and the Western blotting assay. The qPCR results confirmed *DDB2* overexpression and verified that miR-125a-5p and miR-125b-1-5p could reduce the expression of *DDB2* in a synergistic manner (Figure 3C), which was

confirmed by protein detection (Figure 3D). The gray value of Western binds was also calculated (Figure S1).

MiRNA-125a-5p and miR-125b-1-5p induce CRC cell invasion by inhibiting DDB2

DDB2 was reported to inhibit CRC invasion, and we also detected the *DDB2* expression level in primary and metastatic tissues by qRT-PCR. The results showed that *DDB2* was significantly down-regulated in the metastatic niches (Figure S2), which is consistent with previously reported findings.

To evaluate the role of *DDB2* in miR-125-induced colorectal cell invasion, we transfected the cells with miR-125a-5p and miR-125b-1-5p mimics, respectively, based on *DDB2* overexpression. The results revealed that since miR-125a-5p and miR-125b-1-5p increased cell invasion (Figure 4A) by 1.76 and 1.72 folds, respectively (Figure 4B), *DDB2* still suppressed miR-125-induced cell invasion compared to the miR-125-overexpressed colorectal cells (Figure 2A).

Additionally, we transfected cells with miR-125a-5p and the miR-125b-1-5p sponge, respectively, based on *DDB2* depression by small interfering RNA (siRNAs). The results showed that *DDB2* suppression covered miR-125 sponge-reduced cell invasion (Figure 4C), which is consistent with Figure 4D. These results indicate that miRNA-125a-5p and miR-125b-1-5p induce CRC cell invasion by inhibiting *DDB2*.

MiRNA-125a-5p and miR-125b-1-5p activate the CRC cell EMT process via DDB2

It has been reported that *DDB2* blocks EMT-mediated metastasis in CRC, so we detected the effects of miR-125a-5p and miR-125b-1-5p on EMT in the SW480 CRC cell line. The qRT-PCR results showed that miR-125a-5p and miR-125b-1-5p increased the expression of the mesenchymal markers Vimentin (Figure 5A) and N-cadherin (Figure 5B), while simultaneously decreasing that of the epithelial marker E-cadherin (Figure 5C).

Consistently, the Western blot results demonstrated the same results on protein levels (Figure 5D). *DDB2* overexpression suppressed the miR-125a-5p and miR-125b-1-5p-induced EMT process, which is consistent with *DDB2*-reduced colorectal invasion. These results indicated that miR-125a-5p and miR-125b-1-5p could induce EMT by inhibiting *DDB2* expression in CRC cells.

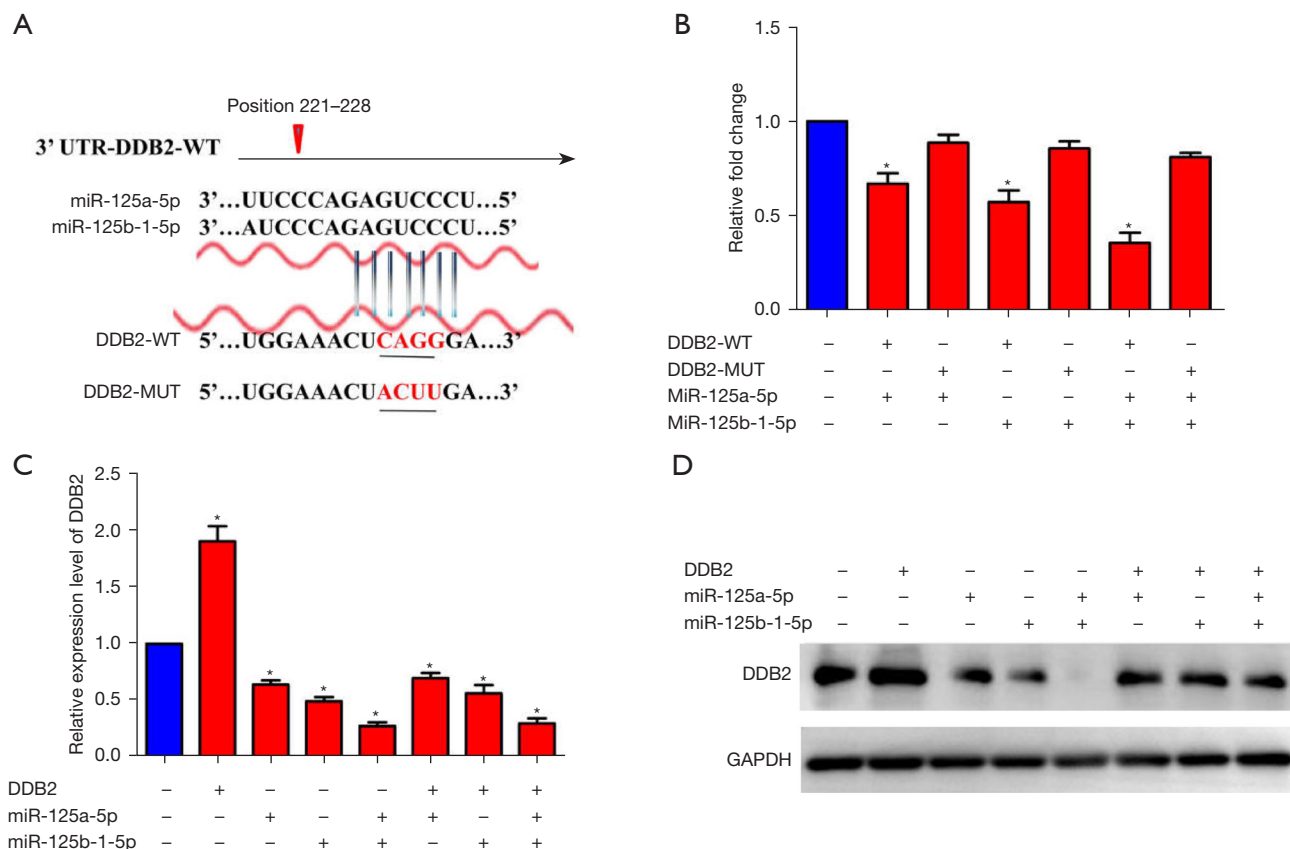


Figure 3 MiRNA-125a-5p and miR-125b-1-5p inhibit *DDB2* expression by binding to the 3'UTR regions of *DDB2* mRNA. *DDB2* is predicted as the co-downstream of miR-125a-5p and miR-125b-1-5p, and the binding position was proposed to be 221–228 located in the 3'UTR region of *DDB2* using the “GAGUCCCU” motif of miR-125a-5p and miR-125b-1-5p. The WT and MUT (CAGG mutated to ACUU) luciferase reporter plasmids of the *DDB2* 3'UTR region were constructed (A). Compared to the *DDB2*-MUT groups, both miR-125a-5p and miR-125b-1-5p could reduce WT *DDB2* expression with a synergistic effect (B). The qPCR results confirmed that miR-125a-5p and miR-125b-1-5p could reduce the expression of *DDB2* in a synergistic manner (C), which was verified by protein detection (D). *, $P < 0.05$. DDB2, damage specific DNA binding protein 2; UTR, untranslated region; WT, wild-type; MUT, mutant-type; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Discussion

CRC is the second most common tumor in the gastrointestinal system. It is associated with high rates of morbidity and mortality, and the incidence of morbidity in young adults is increasing every year, which is a great threat to human health. Unfortunately, the cause of CRC occurrence is still not clear. It is generally believed to be associated with eating habits, with the high consumption of animal fats and proteins as well as low-fiber diets being more likely to cause CRC. Meanwhile, other factors, including genetic susceptibility and pre-cancerous lesions associated with the intestinal tract, are also correlated with

CRC (26,27).

The majority of early-stage patients have no obvious symptoms, and most patients are discovered after tumor metastasis. At present, the major prognostic method for CRC involves the TNM staging system (defined by the Tumor, Node, and Metastasis), which determines the treatment decisions and outcomes of patients with CRC. The purpose of staging is to understand the progression of the tumor, guide the proposed treatment plan, and estimate the prognosis of patients (26,28). The liver is the main target of CRC metastasis, and occurs via miRNA regulation; however, the detailed mechanism has not yet been elucidated (29–31).

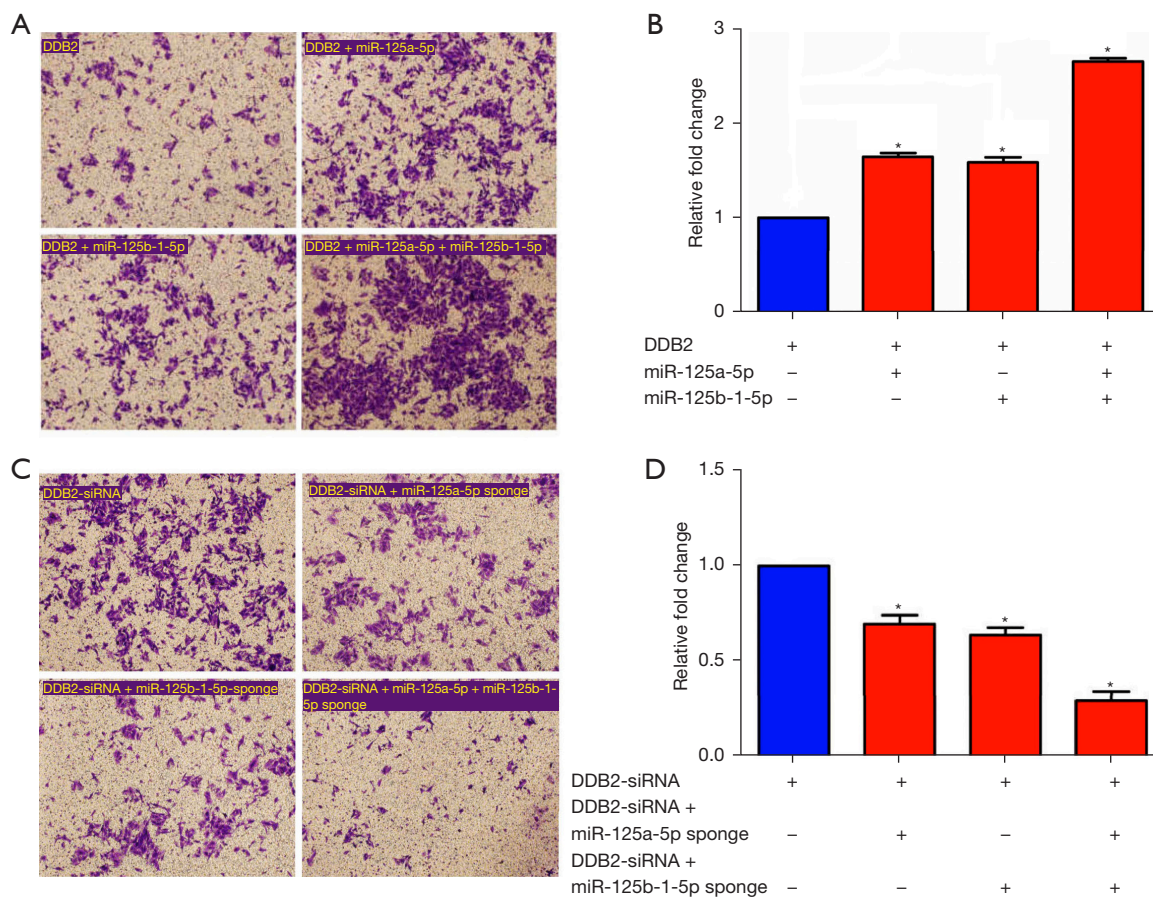


Figure 4 MiRNA-125a-5p and miR-125b-1-5p induced colorectal cancer cell invasion by inhibiting *DDB2*. (A) *DDB2* overexpression suppressed miR-125a-5p- and miR-125b-1-5p-induced cell invasion (crystal violet staining, 100 \times). (B) The invaded cells were statistically analyzed. (C) *DDB2* suppression covered the miR-125 sponge-reduced cell invasion (crystal violet staining, 100 \times). (D) The invaded cells were statistically analyzed. *, $P < 0.05$. *DDB2*, damage specific DNA binding protein 2.

In this study, we found that miR-125a-5p and miR-125b-1-5p were significantly highly expressed in metastatic CRC tissues (Figure 1), indicating miR-125a-5p and miR-125b-1-5p might be correlated with CRC metastasis. We then performed a Transwell filter assay, which confirmed that the overexpression of miR-125a-5p and miR-125b-1-5p could respectively promote the invasion of CRC cells SW480 (Figure 2). Additionally, the combination of miR-125a-5p and miR-125b-1-5p exhibited a synergistic effect on CRC metastasis.

MiRNAs reportedly affect downstream gene expression by binding to the 3'UTR regions of mRNA (32). To explore the potential mechanism behind miR-125a-5p and miR-125b-1-5p-induced cancer invasion, we predicted the downstream genes by cross-analyzing the miRNA target prediction databases. *DDB2* is the target of both miR-

125a-5p and miR-125b-1-5p and is depressed in metastatic colorectal tissues (Figure S2). Next, we utilized a luciferase assay to validate whether *DDB2* is the direct target of miR-125a-5p and miR-125b-1-5p. The results showed that miR-125a-5p and miR-125b-1-5p could bind to the WT *DDB2* but not the MUT, and down-regulate *DDB2* dominantly, indicating that miR-125a-5p and miR-125b-1-5p could bind to the 3'UTR regions of *DDB2* mRNA. *DDB2* has been shown to inhibit colorectal metastasis (33), which is consistent with our findings (Figure 4), illustrating that miR-125a-5p and miR-125b-1-5p increase colorectal cell invasion by down-regulating *DDB2*.

Moreover, *DDB2* is a master suppressor of EMT in CRC. EMT is the process by which epithelial-derived tumor cells transform into stromal cells and acquire the ability to invade and migrate. It exists in multiple

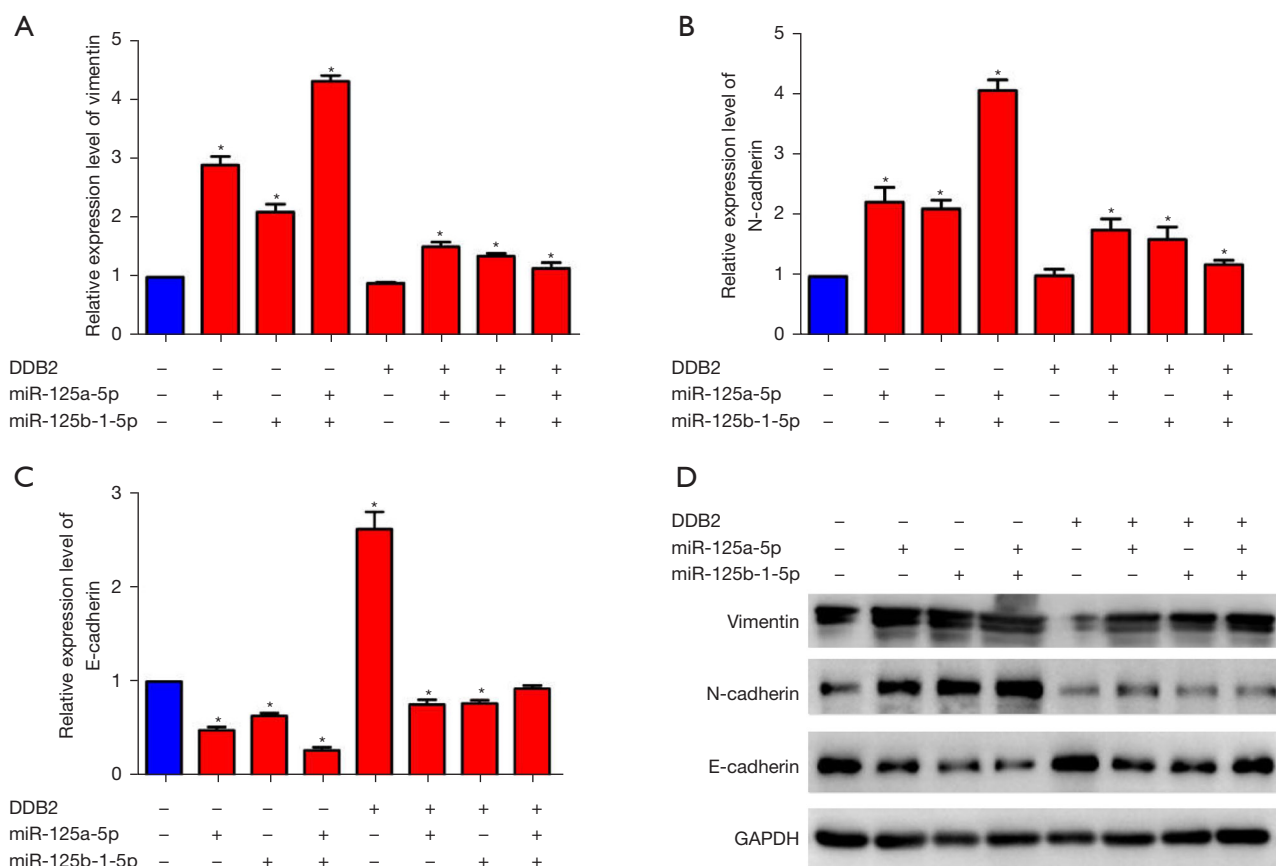


Figure 5 MiRNA-125a-5p and miR-125b-1-5p activated the EMT process in colorectal cancer cells via *DDB2*. MiR-125a-5p and miR-125b-1-5p increased the expression of the mesenchymal markers vimentin (A) and N-cadherin (B), while simultaneously decreasing that of the epithelial marker E-cadherin (C). Consistently, the Western blotting results demonstrated the same results in terms of protein levels. *DDB2* overexpression suppressed the miR-125a-5p- and miR-125b-1-5p-induced EMT process (D). *, $P < 0.05$. *DDB2*, damage specific DNA binding protein 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMT, epithelial-to-mesenchymal transition.

physiological and pathological processes in the human body and plays important roles in tissue remodeling and cancer metastasis. Colorectal epithelial cancer cells lose their polarity through EMT and obtain high interstitial phenotypes, such as migration and invasion. To comprehensively elucidate the mechanism of miR-125a-5p and miR-125b-1-5p-induced colorectal invasion, we detected the effect of miR-125a-5p and miR-125b-1-5p on EMT. The results in *Figure 5* revealed that miR-125a-5p and miR-125b-1-5p activate the EMT process in CRC cells by inhibiting *DDB2*. EMT of malignancy cells increased cancer metastatic ability and chemoresistance (34). Increased EMT is also correlated with the formation of cancer stem cells (CSC) (35). Additionally, accumulating evidence indicates that EMT increased CSC population,

hence resulting CSC-mediated clinical relapse (36). In our study, we found miRNA125a-5p and miRNA125b-1-5p induced epithelial-mesenchymal transition of CRC, so we proposed miRNA125a-5p and miRNA125b-1-5p could increase CSC properties, including chemoresistance and recurrence. The EMT process contributes to increased CSC population through intra-cellular crosstalk, suggesting it may be reasonable to attenuate EMT process for treatment of malignant CRC (36). It seems a promising strategy of blocking miRNA125a-5p and miRNA125b-1-5p expression to inhibit colorectal EMT and CSC for drug development.

It's rarely difficult to obtain enough metastatic tissue for detection of miRNAs, so we collected metastatic niches from kinds of parts, including liver, lung, bladder,

peritoneum, etc. Next step, we will try to analyze the correlation between miRNA125a-5p and miRNA125b-1-5p expression levels and clinical pathology characters, including frequency of liver metastasis, based on enough cases. Meanwhile, it's better to verify the effect of miRNA125a-5p and miRNA125b-1-5p on CRC metastasis *in vivo*, and use multiple methods to assess the effect of miRNA125a-5p and miRNA125b-1-5p on cell invasion and migration.

Conclusions

Our results indicated that miR-125a-5p and miR-125b-1-5p could activate the EMT process by down-regulating DDB2, and thus, enhance the metastatic ability of CRC cells. Our findings preliminarily explored the mechanism of CRC metastasis, which may provide helpful information for future treatment.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The methods were performed in accordance with the approved guidelines by the Ethics Committee of The First Affiliated Hospital of Nanjing Medical University (No. 2010-SR-091). Informed consent

was obtained from all subjects.

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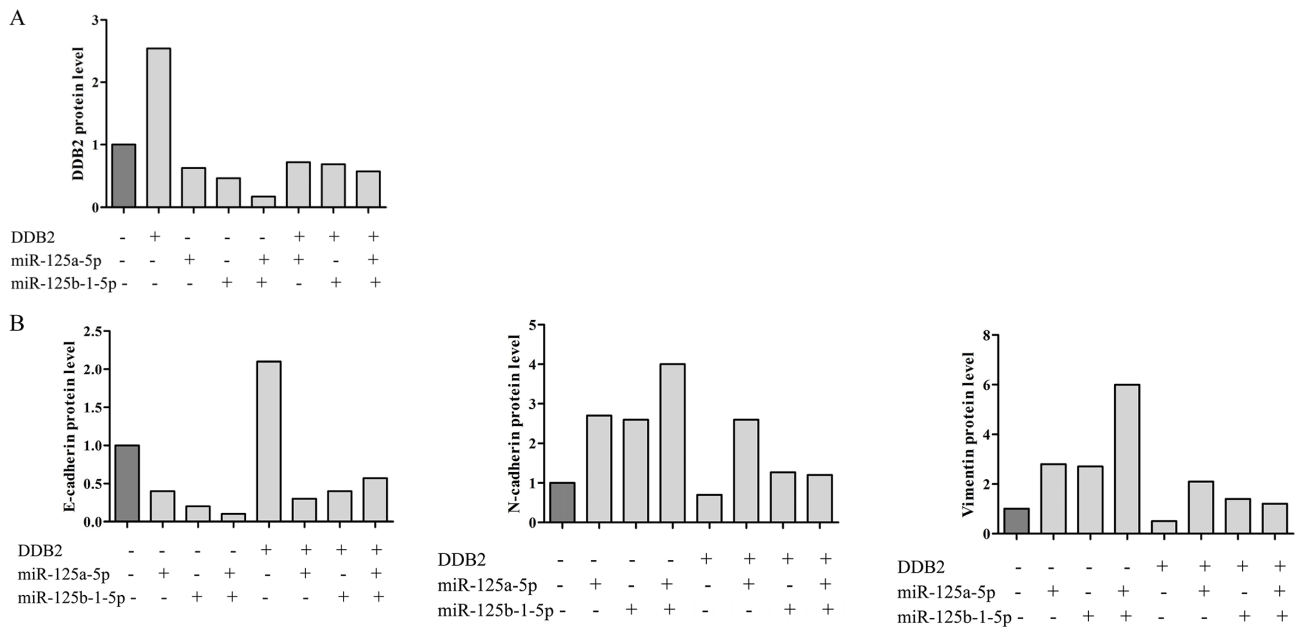


Figure S1 Gray value of the Western blotting binds. (A) Grayscale values of the protein bands in *Figure 3D*. (B) Grayscale values of the protein bands in *Figure 5D*. DDB2, damage specific DNA binding protein 2.

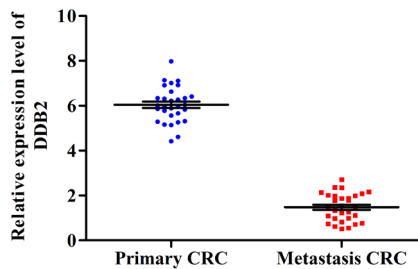


Figure S2 Expression level of *DDB2* is dominantly reduced in metastasis niches compared to primary tissues. CRC, colorectal cancer; DDB2, damage specific DNA binding protein 2.

Table S1 Primer sequences for qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
miRNA125a-5p	AGCCGCTTAAAGGGTCT	GTGCAGGGTCCGAGGT
miRNA125b-1-5p	AGCCGCTCACAAGTTAG	GTGCAGGGTCCGAGGT
miRNA125b-2-5p	AGCCGCAAGAGCCTGAC	GTGCAGGGTCCGAGGT
<i>U6</i>	CTCGCTTCGGCAGCAC	AACGCTTCACGAATTTGCGT
<i>DDB2</i>	AGGACCCTCCACCAGCATAA	CAGGCTGCCCTTTGATGA
<i>GAPDH</i>	GAGAAGGCTGGGGCTCATT	AGTGATGGCATGGACTGTGG

DDB2, damage specific DNA binding protein 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction.