

Original Article

MicroRNA-103a inhibits proliferation, migration and invasion in colorectal cancer cells

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Abstract: Colorectal cancer (CRC) is the second leading cause of cancer-related death in the western country. MiR-103a is an important post-transcriptional regulator, which has been validated as a tumor suppressor in many human cancers. However, the detailed role of miR-103a in CRC remains to be elucidated. In our study, real-time RT-PCR was performed to detect the expression levels of miR-103a in CRC cell lines and clinical CRC specimens. To further explore the potential role of miR-103a, we restored its expression in CaCO-2 and SW480 cell lines by transfection with miR-103a mimics. Effects of miR-103a on cell proliferation, migration and invasion were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), wound scratch and trans-well assays. The results showed that miR-103a was significantly down-regulated in both CRC cell lines ($P < 0.05$) and clinical specimens ($P < 0.05$). Decreased expression of miR-103a significantly correlated with lymph node metastasis ($P < 0.05$) and local invasion ($P < 0.05$). Kaplan-Meier survival analysis showed that the CRC patients with low miR-103a levels had a significantly poorer prognosis than those with the high miR-103a levels ($P < 0.05$). Multivariate analysis revealed that miR-103a expression ($P < 0.05$) and lymph node metastasis ($P < 0.05$) could be independent prognostic indicators for overall survival rates of CRC patients. Furthermore, gain-of-function experiment indicated that reintroduction of miR-103a significantly reduced CRC cells growth, migration and invasion (All $P < 0.05$). In conclusion, our observations suggest that miR-103a has potential as a prognostic biomarker and functions as a tumor suppressor in CRC.

Keywords: Colorectal cancer, miR-103a, prognosis, tumor suppressor

Introduction

Colorectal cancer (CRC) is the third most common malignancies and the fourth leading death-related cancers worldwide [1]. Despite innovative therapeutic strategies applied to CRC treatment, nearly 50% of CRC patients show tumor recurrence and the prognosis has not significantly changed in the last 20 years [2]. Most recurrences of CRC are thought to be the result of tumor growth and metastasis of cancer cells [3]. Therefore, understanding the molecular mechanisms of recurrence in CRC is of crucial significance to the development of therapeutic strategies for CRC patients.

MicroRNAs (miRNAs) are a new classification of small, endogenous, single-stranded RNAs composed of 19-24 nucleotides, which modulate gene expression by binding to the 3'-untranslated region (3'-UTR) of the target gene mRNA

[4, 5]. MiRNAs play important regulatory roles in diverse cellular processes, including growth, cell cycle, invasion, migration and apoptosis [6]. Numerous studies have demonstrated miRNAs function as an oncogene and/or a tumor suppressor during the development and progression of human cancer [7, 8]. For example, Zhi et al reported that miR-494 inhibits proliferation and metastasis of osteosarcoma through repressing insulin receptor substrate-1 [9]. Wang et al showed that upregulation of miR-524-5p enhances the cisplatin sensitivity of gastric cancer cells by modulating proliferation and metastasis via targeting SOX9 [10]. Hu et al demonstrated that miR-497 enhances metastasis of oral squamous cell carcinoma through SMAD7 suppression [11]. Tsai et al found that miR-26b inhibits tumor metastasis by targeting the KPNA2/c-jun pathway in human gastric cancer [12].

miR-103a inhibits cell proliferation, migration and invasion

Recently, miR-103a is found to be desregulated in a variety of human cancers [13]. Accumulating evidence has suggested that miR-103a serve to affect a novel tumor suppressor in human cancers. Liang et al found that miR-103a inhibits gastric cancer cell proliferation, migration and invasion by targeting c-Myb [14]. However, little knowledge is known about the potential prognostic value and the precise function of miR-103a in CRC. In this study, we investigated the expression of miR-103a in clinical CRC tissues and cell lines, and the correlation between miR-103a levels and clinical characteristics was also analyzed. Further studies in vitro showed that the restoration of miR-103a inhibited CRC cells growth, migration and invasion.

Material and methods

Cell culture and cell culture

Human CRC cell lines (CaCO-2 and SW480) and a normal colon epithelium cell line (FHC) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator at 37°C with an atmosphere of 5% CO₂.

Cell transfection

MiR-103a mimics and mimics control (NC) were purchased from GenePharma (Shanghai, China). Transfection was performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Briefly, approximately 24 h before cell transfection, 2.5×10⁶ CaCO-2 and SW480 cells were seeded in each well of 6-well plates. The cells with 55~75% confluence were then transiently transfected with 50 pM miR-103a mimics or NC. Transfection efficiency was detected in every experiment by real-time RT-PCR assay.

CRC sample collection

A total of 45 CRC tissues and adjacent non-cancerous tissues (>5 cm from tumor tissue margin) were collected from the Department of General Surgery, Tianjin Tumor Hospital (Tianjin, China) between December 2006 and December 2014. This research was approved by the eth-

ics committee of Tianjin Tumor Hospital. The written informed consent was provided from each subject or the legal representative. All the tissues were flash-frozen in liquid nitrogen immediately and stored at -80°C until further experiment. The CRC samples were carefully examined by two pathologists, and the staging of each patient was evaluating according to the 7th edition of the UICC TNM staging system [15]. The median follow-up period of patients enrolled in this study was 60 months. Overall survival (OS) was defined as the time from the day of operation to death or the date of last follow-up.

RNA preparation, cDNA synthesis and real-time RT-PCR

Total RNA from CRC tissues and cells was prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The First Strand cDNA was synthesized by using a miScript Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Real-time RT-PCR assay was conducted by using an ABI 7100 real-time PCR system (Applied Biosystems, Foster City, CA, USA). In brief, total 20 µl mixtures with 10 µl of 2×miScript SYBR Green, 1 µl of DNA, 1 µl of primers and 8 µl of RNase-free water. The reaction was initially denatured 95°C for 10 min, followed by 45 cycles of denaturation at 98°C for 10 sec, annealing and extension at 60°C for 30 sec. The data were analyzed using the comparative Ct method [16]. The primers used for the amplification were as follows: U6: 5'- CTCGCTTCGGCAGCACA -3' (forward), 5'- AACGCTTCACGAATTTGCGT -3' (reverse); miR-103a: 5'- AGCAGCATTGTACAGGGCTATGA -3' (forward), universal Primer (reverse).

Cell proliferation assay

CaCO-2 and SW480 cells were transfected as above description. The transfected cells (4×10³ cells/well) were plated into 96-well plates. At 0, 24, 48 and 72 h post-transfection, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) was added into each well and cultured for 6 h at 37°C, followed by removal of the culture medium and the addition of 150 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). The value of optical density (OD) was determined at 480 nm (with 630 nm as the reference wavelength) by a Multiskan FC Micro-

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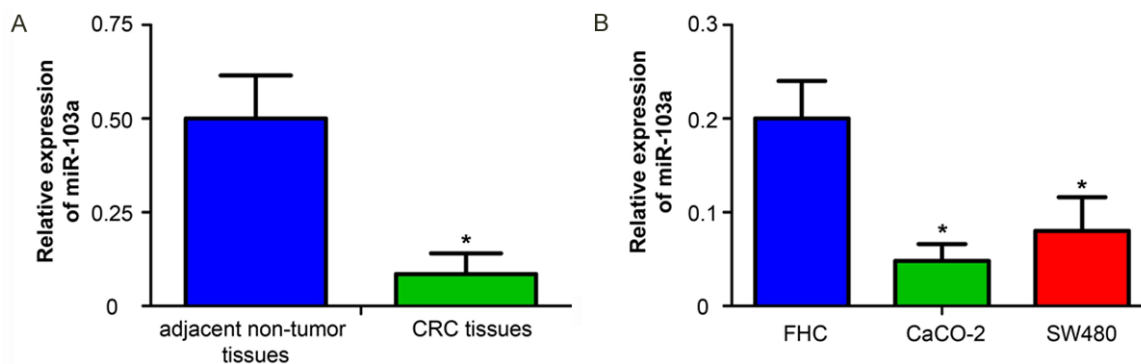


Figure 1. MiR-103a is downregulated in CRC tissues and cell lines. A. Relative expression of miR-103a in 45 paired of CRC tissues and adjacent non-tumor tissues. * $P < 0.05$ vs. adjacent non-tumor tissues. B. MiR-103a expression levels in CRC cell lines (CaCO-2 and SW480) and a normal colon epithelium cell line (FHC). MiR: microRNAs; CRC: colorectal cancer. * $P < 0.05$ vs. FHC.

plate Photometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Wound healing assay

Transfected cells (4×10^6) were seeded into 6-well plates for 6 h. Thereafter, a linear wound of cellular monolayer was created in the confluent cells. After wounding, the debris was removed by washing the cells with PBS. Migration of cells into the wound was observed at 0 and 12 h after transfection by using an Olympus IX51 inverted microscope (Olympus, Tokyo, Japan).

Cell invasion assay

Cell invasion assay was performed using a Transwell system (Corning, Lowell, MA, USA) according to the manufacturer's protocols. Briefly, 2×10^5 post-transfected cells were seeded into the upper chamber coated with extracellular matrix (BD Biosciences, Bedford, MA, USA). The low chamber was filled with 500 μ l RPMI 1640 medium containing 10% FBS as a chemoattractant. After incubation for 48 h, cells on the lower side of the filter were fixed in 70% ethanol (Sigma-Aldrich, St. Louis, MO, USA) for 30 min and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 10 min on a glass slide. The number of cells in five randomly selected fields was counted under an Olympus IX51 inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data are presented as the mean \pm standard deviation (SD). Statistical analyses were con-

ducted with SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Each experiment was repeated at least three times. The differences between two groups were evaluated by using the Student's t-test or one-way ANOVA. Chi-square (χ^2) test was used for expression correlation analysis. The Kaplan-Meier method and log-rank test were used to estimate differences in survival between two groups. $P < 0.05$ were considered to be statistically significant.

Results

MiR-103a is downregulated in CRC tissues and cell lines

To determine the role of miR-103a in CRC progression, we examined its expression in CRC tissues and adjacent non-cancerous tissues by real-time RT-PCR. We found that miR-103a was downregulated in the CRC tissues compared with the paired adjacent non-cancerous tissues (**Figure 1A**, $P < 0.05$). We then analyzed the expression levels of miR-103a in CRC cell lines (CaCO-2 and SW480) and a normal colon epithelium cell line (FHC). Interesting, the results showed that miR-103a expression was also decreased in the CaCO-2 and SW480 cells compared with the FHC (**Figure 1B**, $P < 0.05$).

Decreased expression of miR-103a is associated with lymph node metastasis and local invasion

To investigate the clinical relevance of miR-103a in CRC, we divided the 45 patients to the high miR-103a expression group ($n=16$) and low miR-103a expression group ($n=29$) by using

Table 1. Relationship between clinical parameters and miR-103a expression in patients with CRC

Variable	Cases	miR-103a expression		P value
		Low (29, %)	High (16, %)	
Gender				0.175
Male	23	17 (37.8)	6 (13.3)	
Female	22	12 (26.7)	10 (22.2)	
Age (y)				0.373
<60	18	13 (28.9)	5 (11.1)	
≥60	27	16 (35.6)	11 (24.4)	
Local invasion				<0.001
T1~T2	15	3 (6.7)	12 (26.7)	
T3~T4	30	26 (57.8)	4 (8.9)	
Tumor size				0.062
≤5 cm	28	15 (33.3)	13 (28.9)	
>5 cm	17	14 (31.1)	3 (6.7)	
Histologic grade				0.102
Well+moderate	13	6 (13.3)	7 (15.6)	
Poor	32	23 (51.1)	9 (20.0)	
TNM stage				0.157
I+II	19	10 (22.2)	9 (20.0)	
III+IV	26	19 (42.2)	7 (15.6)	
Lymph node metastasis				0.023
Absent	29	15 (33.3)	14 (33.1)	
Present	16	14 (31.1)	2 (4.4)	

CRC: colorectal cancer; miR: microRNAs; TNM: Tumor Node Metastasis.

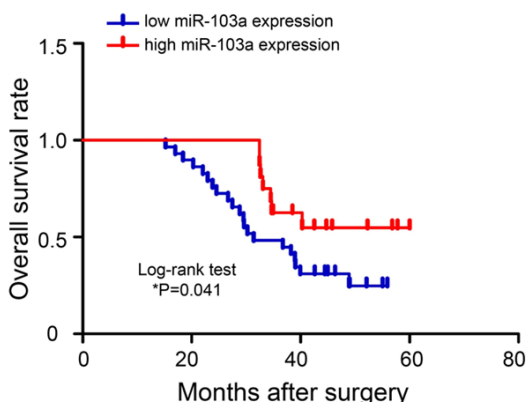


Figure 2. Kaplan-Meier overall survival curves of patients with CRC based on miR-103a expression. Patients in the low miR-103a expression group had significantly lower overall survival times than those in the high miR-103a expression group. *P<0.05 vs. high miR-103a expression.

the mean value (0.76) of relative expression levels as a cutoff. The correlation between the miR-103a expression levels and clinicopathological characteristics of CRC was shown in

Table 1. Our data showed that decreased expression of miR-103a was associated with lymph node metastasis (P<0.05) and local invasion (P<0.05). However, no significant correlations were observed between miR-103a expression and other clinical features including age, gender, tumor size, TNM stage and histologic grade. Collectively, these observations suggested that down-regulation of miR-103a may play a key role in the progression of CRC.

MiR-103a is a prognostic factor for overall survival of CRC patients

Of the 45 patients, 28 were died during the follow-up period. The 5-year overall survival rate was 37.8%. Kaplan-Meier survival analysis showed that CRC patients with low miR-103a levels had a significantly poorer prognosis than those with high miR-103a levels (**Figure 2**, P<0.05). In addition, cox regression multivariate analysis revealed that miR-103a expression (P<0.05) and lymph node metastasis (P<0.05) maintained as independent prognostic indicators for overall survival rates of CRC patients (**Table 2**).

Effect of miR-103a over-expression on cell proliferation

As miR-103a was decreased in CRC tissues and cell lines, we transfected miR-103a mimics or mimics control (NC) into CaCO-2 and SW480. The results from real-time RT-PCR assay demonstrated that miR-103a mimics significantly upregulated the levels of miR-103a in CaCO-2 and SW480 cells compared to NC treated CaCO-2 and SW480 cells (**Figure 3A**, P<0.05). MTT assay was performed to determine miR-103a effect on proliferation in CRC cells. It was found that overexpression of miR-103a significantly inhibited cell proliferation in CaCO-2 and SW480 cells (**Figure 3B**, P<0.05).

Overexpression of miR-103a inhibits cell migration of CRC

The above data showed that miR-103a expression is correlated with the metastatic ability of

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Table 2. Univariate and multivariate analysis of clinicopathological factors for overall survival of CRC patients

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Gender (male vs. female)	1.525 (0.864-2.334)	0.435		
Age (<60 vs. ≥60)	1.176 (0.632-2.257)	0.584		
Local invasion (T1~T2 vs. T3~T4)	0.623 (0.217-1.456)	0.162		
Tumor size (≤5 cm vs. >5 cm)	1.543 (0.769-2.682)	0.255		
Histologic grade (well+moderate vs. poor)	0.661 (0.314-1.078)	0.495		
TNM stage (I+II vs. III+IV)	1.441 (0.594-2.404)	0.307		
Lymph node metastasis (absent vs. present)	3.929 (1.524-9.257)	0.001	2.464 (1.021-6.577)	0.027
miR-103a expression (low vs. high)	2.638 (1.441-6.078)	0.007	1.578 (1.015-4.765)	0.038

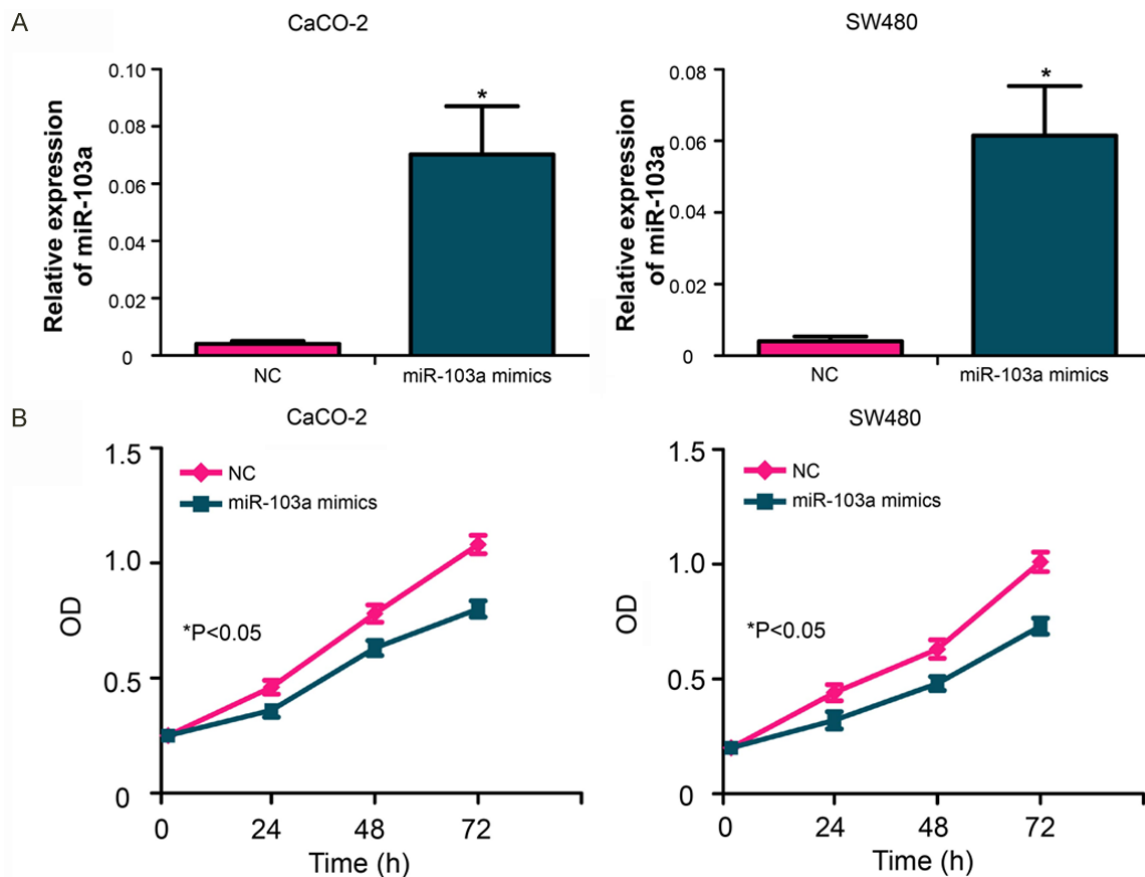


Figure 3. MiR-103a overexpression inhibited CRC cells proliferation in vitro. A. Expression of miR-103a in CaCO-2 and SW480 cells was detected by real-time RT-PCR after transfected with miR-103a mimics or mimics control (NC). B. MiR-103a overexpression inhibited CRC cells proliferation ability. *P<0.05 vs. NC.

CRC cells. Thus, to investigate the miR-103a effect on migration, wound healing assay was performed in CaCO-2 and SW480 cells after transfected with miR-103a mimics or NC. Consistent with our speculation, overexpression of miR-103a in CRC cells significantly inhibited cell migration at 12 h after scratching (Figure 4, P<0.05).

Restoration of miR-103a suppresses cell invasion of CRC

To further verify the role of miR-103a effect on CRC cells invasion, we used in vitro transwell assay. We found that CaCO-2 cells treated with miR-103a mimics had less invasive ability than NC treated CaCO-2 cells (Figure 5A, P<0.05).

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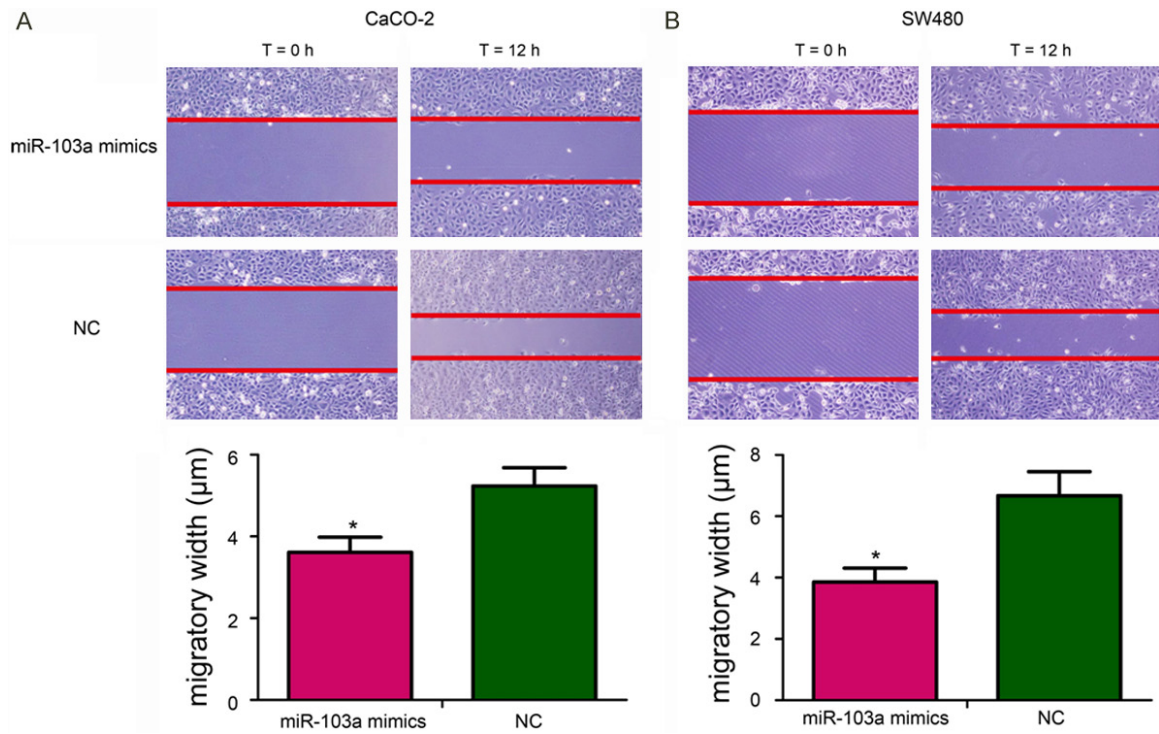


Figure 4. Overexpression of miR-103a inhibits cell migration of CRC in vitro. A. Cell migration of CaCO-2 cells transfected with miR-103a mimics or NC was determined by wound-healing assay. B. miR-103a mimics treatment was able to inhibit the migration of SW480 cells. The red lines indicate the migration front. Representative photographs were shown (magnification, 200×). *P<0.05 vs. NC.

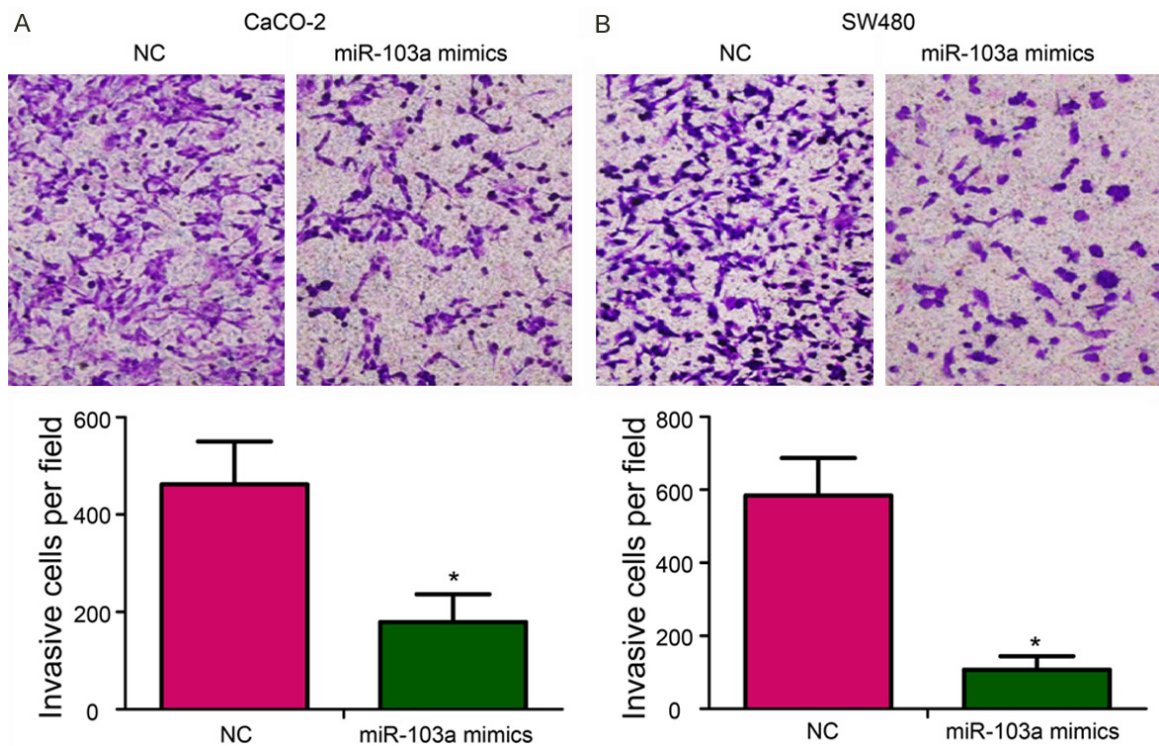


Figure 5. Restoration of miR-103a suppresses cell invasion of CRC. Transwell analysis of CaCO-2 (A) and SW480 (B) cells after treatment with miR-103a mimics or NC, and the number of invasive cells per field is shown below. Representative photographs were shown (magnification, 200×). *P<0.05 vs. NC.

Similarly, transwell assay with Matrigel showed that the invasive ability was significantly suppressed in SW480 cells transfected with miR-103a mimics when compared to NC transfected cells (**Figure 5B**, $P < 0.05$). Collectively, these results suggested that miR-103a can efficiently inhibited growth, migration and invasion of CRC cells.

Discussion

CRC has been described as a multistep malignancy due to the progressive accumulation of chromosomal rearrangements and mutations involving critical tumor suppressors and/or oncogenes [17]. Emerging evidences have indicated that miRNAs can play key regulatory roles in gene expression associated with human cancer development and progression [18, 19]. Recently, multiple of studies revealed that miRNAs act as an effective biomarker for the cancer diagnosis, prognosis and therapy [20]. A series of studies have been underway for CRC, and some cancer-related miRNAs have been identified. For example, down-regulation of miR-24-3p has a potential role in prognosis and contributes to the development and progression of CRC [21]. MiR-205 acts as a tumor suppressor in CRC through targeting cAMP responsive element binding protein 1 (CREB1) [22]. MiR-9 represses cell migration and invasion by downregulation of TM4SF1 in CRC [23].

The aberrant expression of miR-103a is a frequent event in various kinds of human cancers including gastric cancer [14], bladder cancer [13] and lung adenocarcinoma [24], suggesting that miR-103a play an important role in the tumorigenesis. However, the clinical significance and roles of miR-103a in CRC remain unknown. In the present study, to our knowledge, we first reported that miR-103a was downregulated in CRC samples from patients compared with adjacent non-cancerous tissues. Downregulation of miR-103a expression was correlated with lymph node metastasis and local invasion.

To further explore the potential prognostic role of miR-103a in CRC patients, we investigated the relationship between miR-103a expression and overall survival. Kaplan-Meier survival analysis showed that the patients with low miR-103a expression had a poorer survival compared those with high miR-103a expression.

Cox regression multivariate analysis indicated that miR-103a expression and lymph node metastasis could be served as independent prognostic indicators for CRC patients. All these data demonstrated that down-regulation of miR-103a in CRC was associated poor prognosis.

Accumulating evidence firmly demonstrates that miRNAs control various key cellular processes such as growth, metastasis and apoptosis [6]. Liang et al found that miR-103a functions as a tumor suppressor in gastric cancer regulating cellular proliferation, migration and invasion [14]. Functional studies were also performed to analyze the role of miR-103a on behavior of CRC cells. In agreement with the previous study, we found that the overexpression of miR-103a in CRC cells significantly inhibited cell proliferation, migration and invasion. These results indicated that miR-103a act as a tumor suppressor in CRC and restoration of miR-103a might provide a therapeutic strategy for CRC.

In conclusion, the present study demonstrated that miR-103a expression is downregulated in CRC tissues and cell lines and is inversely correlated with lymph node metastasis and local invasion. MiR-103a functions as tumor suppressor in CRC inhibiting cell growth and metastasis. These finding suggested that miR-103a may serve as a new prognostic and therapeutic agent for CRC.

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Disclosure of conflict of interest

None.

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