European Review for Medical and Pharmacological Sciences 2016; 20: 4682-4687

Down-regulation of microRNA-143 is associated with colorectal cancer progression

J.-W. BAI[1], H.-Z. XUE[2], C. ZHANG[1]

[1]Department of Gastrointestinal Surgery, People's Hospital of Zhengzhou University, Zhengzhou, China
[2]Department of Hepatobiliary and Pancreas Surgery, People's Hospital of Zhengzhou University, Zhengzhou, China

Abstract. – OBJECTIVE: Colorectal cancer (CRC) is one of the most prevalent carcinomas worldwide. Tumor metastasis and recurrence are leading causes of CRC-related deaths. Given the role of microRNA (miRNA) in CRC invasion and metastasis, we explored the association between miRNA-143 expression and clinicopathologic characteristics in CRC, as well as the effects of miRNA-143 on CRC cell invasion *in vitro*.

MATERIALS AND METHODS: Quantitative real-time PCR was conducted to assess the expression of miR-143 in tissue specimens and cell lines. CCK-8 test and transwell assay were conducted to explore the effects of miR-143 on the proliferation and invasion of CRC cell lines, respectively. Luciferase reporter assay and transfection technique were used to validate the correlation between miR-143 and epidermal growth factor receptor 3 (ERBB3) in CRC.

RESULTS: miR-143 was down-regulated in human CRC tissues, its expression negatively correlated with CRC metastasis. miR-143 negatively regulated ERBB3 expression by directly targeting its 3'UTR in human colorectal cancer cells. Overexpression of miR-143 inhibited CRC cells invasion but did not affect its proliferation *in vitro*. Knockdown of ERBB3 also significantly suppressed CRC cells invasion.

CONCLUSIONS: These data suggest that miR-143 suppressed the invasion and metastasis ability by suppressing ERBB3 expression in CRC cells, providing a novel and promising therapeutic target for the prevention and treatment of CRC.

Key Words:
miR-143, ERBB3, Invasion, Colorectal cancer.

Introduction

Colorectal cancer (CRC) is one of the most prevalent carcinomas in the world. Its distant metastasis is the leading cause of cancer-related death in CRC patients[1]. Colorectal carcinogenesis is a complex multistage process involving multiple genetic changes and alterations in various molecular signaling pathways[2,3]. Although diagnosis and treatment of CRC have achieved much progress with the improvement of surgical and medical treatments, approximately 30-50 % of CRC patients develop local tumor recurrence or distant metastasis after curative resection of the primary lesion[4,5]. Therefore, the underlying molecular mechanism of CRC metastasis progression is in urgent demand.

Recently many epigenetic events are widely investigated in the cancer development. Aberrant expression of microRNAs (miRNAs) is frequently reported in various types of cancers[6]. MiRNAs are small, noncoding RNA molecules of approximately 19-25 nucleotides that potentially regulate 20-30% of gene expression[7]. Mature miRNAs play important regulatory roles in cell growth, invasion, differentiation and cell death[8,9]. According to their targets, miRNAs are divided into onco-miRNAs and tumor suppressors in cancers[10]. In recent years, certain aberrant miRNAs have been implicated in CRC development[11,12]. However, it is urgently essential to reveal how miRNAs act within the context of the molecular mechanisms for metastasis in CRC.

In this study, we compared the expression levels of miR-143 in primary CRC and adjacent non-tumor tissues. Ectopic expression of miR-143 inhibited the invasion of CRC cell lines by targeting epidermal growth factor receptor 3 (ERBB3) without alteration of cell proliferation. Based on our results, we suggested that miR-143 inhibited CRC metastasis in part by regulation of the ERBB3 pathway.

Materials and Methods

Clinical CRC Specimens

Eighty of primary colorectal cancer tissues and eighteen pairs of primary colorectal cancer tissues and matched non-tumor tissues were collected

Corresponding Author: Huanzhou Xue, MD; e-mail: xue_huanzhou@sina.com

from patients who underwent curative surgical resection at the department of Surgery, People's Hospital of Zhengzhou University (Zhengzhou, China) from 2014 to 2016. None of the patients received preoperative treatment, such as radiation therapy or chemotherapy. Written consent of tissue donation for research purposes was obtained from each patient. The study protocol was approved by the Scientific and Ethical Committee of Zhengzhou University.

Cell Lines and Cell Culture

Human colorectal cancer cell lines (HCT116 and SW480) used in the present study were purchased from Cell Center of Shanghai Institute of Life Science, Chinese Academy of Science (Shanghai, China). All Cells were grown in DMEM (Gibco, Grand Island, NY, USA) with 10% FBS (Hyclone, Loran, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and incubated at 37°C with 5% CO_2.

Cell Transfection

The miR-143 mimics and a non-specific miR-control were synthesized and purified by Genephama Biotech (Shanghai, China). The sequences of miR-143 were: Sense: 5'-GUGAAAUGUUUAGGACCACUAG-3', Anti-sense: 5'-CUAGUGGUCCUAAAC

AUUUCAC-3', A non-specific miR with no homology to any known human miRNA was used as negative control. The target ERBB3 sequence of the siRNA was AAGAGCGACTAGACATCAAGC. A scrambled siRNA (Invitrogen, Carlsbad, CA, USA) was included as a negative control. Cells (HCT116, SW480) cultured in 6-well cell culture plate were seeded 24 hrs before transfection. A final concentration of 50 nM of RNA mimics or 100 nM of siRNA and their respective controls were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 or 48 h, cells were harvested for further experiments.

RNA Extraction and SYBR Green Quantitative PCR Analysis

Total RNA from tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). MiR-143 expressions in tissues were detected using a Platinum SYBR Green qPCR SuperMix-UDG system (Invitrogen). Expression of RNU6B was used as an internal control. Gene expression was normalized to internal controls and fold changes were calculated using $2^{-\Delta\Delta}$CT method.

Cell Proliferation Assay

A Cell Counting Kit-8 (CCK-8) was used to estimate cell proliferation ability. In all, 3×10^3 cells were seeded in each 96-well plate for 24 h, transfected with the indicated miRNA or control, and further incubated for 48 h. 10 μl of CCK-8 reagent was added to each well at 1h before the endpoint of incubation. Absorbance was measured at a wavelength of 450 nM using a spectrophotometer (Multiskan MK3, Thermo Electron Corporation, Waltham, MA, USA).

Cell Invasion Assay

Cells were transfected with a miR-143, siRNA and their respective controls. After 24 hrs, invasion assay were performed with modified Boyden chambers containing filter inserts coated with Matrigel (24-well insert; pore size, 8 μm; BD Biosciences, Franklin Lakes, NJ, USA). The cells were incubated for 16 hrs and cells invaded on the lower surface of the membrane were stained. The data are expressed as means ± SEM from 3 independent experiments.

Vectors Construction and Reporter Assays

For confirmation of direct target binding, the 3'UTR of ERBB3 identified by TargetScan (http://www.targetscan.org) was cloned into a REPORT vector system (GeneCopoeia, Rockville, MD, USA). Cells were seeded in 96 well plates and co-transfected with miRNA precursor or negative control and vector system, as well as renilla vector (pRL-TK) using lipofectamine 2000 (Invitrogen). Luciferase activity was measured using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well.

Western Blot Analysis

Protein lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membrane was incubated with a rabbit antibody against human ERBB3 (Upstate Biotechnology, Lake Placid, NY, USA) and detected by chemiluminescence. GAPDH (Cell Signaling Technology, Inc., Danvers, MA, USA) was used as a protein loading control. Changes in ERBB3 and GAPDH protein levels were quantified by scanning densitometry (model GS 670; Bio-Rad, Hercules, CA, USA).

Table I. Correlation of miR-143 expression with clinical characteristics of colorectal cancers.

		miR-143 expression		
		Low (n=58)	High (n=22)	*p*-value
Gender	Male	33	10	0.359
	Female	25	12	
Age	≤60	30	9	0.388
	>60	28	13	
Tumor size (cm)	≤ 3	32	11	0.679
	>3	26	11	
Differentiation	Well	11	8	0.256
	Moderate	25	8	
	Poor	22	6	
pT stage	T1/T2	24	13	0.156
	T3/T4	34	9	
Metastasis lymph node	Yes	39	6	0.0013
	No	19	16	

Statistical Analysis

All values were expressed as means ± SEM. Statistical analysis was performed by 2-tailed Student's *t*-test, and results were considered statistically significant at $p<0.05$. The correlation between miR-143 and clinicopathologic characteristics was performed by Pearson χ^2-analysis. $p<0.05$ were considered to be statistically significant.

Results

MiR-143 is frequently downregulated in CRC tissues. We firstly investigated the role of miR-143 in colorectal cancer occurrence by detecting its expression in 18 pair of cancer specimens and non-tumor tissue specimens. Our results showed that miR-143 expression is significantly ($p<0.05$) decreased in cancer tissues when compared with that in the paired adjacent non-tumor tissues (Figure 1). Moreover, we further analysis the correlation of miR-143 expression levels with clinicopathologic characteristics of colorectal cancer patients using 80 cancer specimens. We observed that miR-143 was downregulated in CRC tissues from metastatic cases compared with non-metastatic cases and correlated significantly with cancer metastasis (Table I). These results indicated that miR-143 expression was negatively correlated with occurrence and metastasis of CRC. MiR-143 inhibits CRC cell invasion but not affects its proliferation.

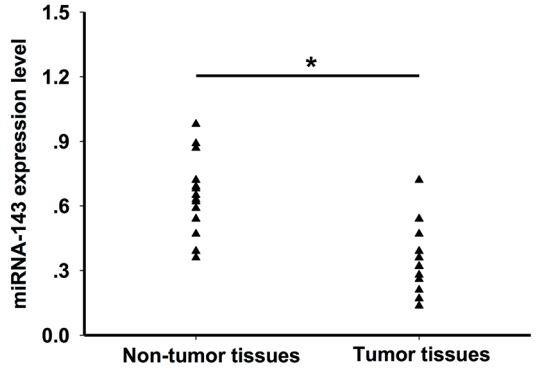

Figure 1. MiR-143 was down-regulated in CRCs. Expression level of miR-143 in each individual case of CSCs, adjacent non-tumor tissues was examined using qRT-PCR. U6 was used as an internal control. Data were presented as means ± SEM. *Different ($p < 0.05$) from non-tumor control.

To Investigate if miR-143 Regulated Colorectal Cancer Growth

We performed proliferation and invasion assays by transfecting miR-143 mimics or miR-control into HCT116 and SW480 cells. After transfection, the miR-143 is highly increased in comparison with miR-control in these cell lines measured by qRT-PCR (Figure 2A). There was no significant change in cell proliferation in the miR-143-transfected cells (Figure 2B). However, overexpression of miR-143 significantly suppressed ($p<0.05$) cell invasion compared with miR-control in both HCT116 and SW480 cells (Figure 2C).

MiR-143 Directly Targets ERBB3

Using online miRNA target prediction databases, we predicted ERBB3 as a target of miR-143. Then luciferase reporter assay we used to confirm whether ERBB3 was a direct target of miR-143. Our data showed that the luciferase activity of pGL3-ERBB3-3'UTR reporter was significantly suppressed in miR-143-transfected cells than in NC-transfected cells (Figure 3A). Further study tested that overexpression of miR-143 significantly decreased ERBB3 protein expression (Figure 3B). These findings indicated that ERBB3 was negatively regulated by miR-143 in colorectal cancer cells.

Knockdown of ERBB3 Inhibits Colorectal Cancer Invasion in vitro

To confirm the above findings, the expression of ERBB3 was knocked down by transfection with siRNA. Western blot analysis showed that the expression levels of ERBB3 protein were decreased after transfection of ERBB3 siRNA when

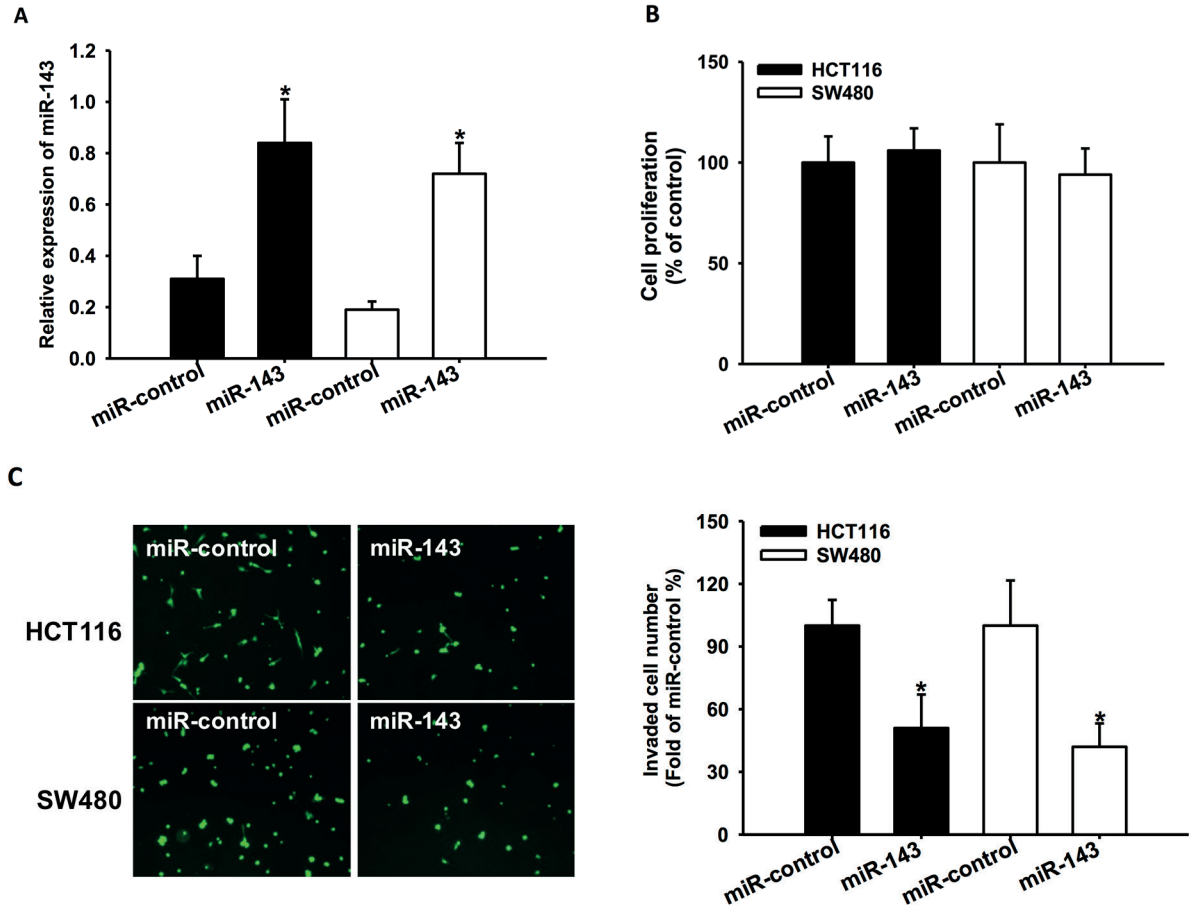

Figure 2. Overexpression of miR-143 inhibited CRC cell invasion, but not cell proliferation. ***(A)*** The expression of miR-143 was increased after transfected with miR-143 or miR-control for 48 hrs assessed by qRT-PCR. ***(B)*** Overexpression of miR-143 did not affect cell proliferation determined by CCK-8 Assay. ***(C)*** Overexpression of miR-143 significantly suppressed cell invasion detected by transwell assay. Data were represented as a percentage of cell number relative to the miR-control (100%) from 4 independent experiments. *Different ($p < 0.05$) from miR-control.

compared with negative control in both HCT116 and SW480 cells (Figure 4A). A transwell invasion assay showed that knockdown of ERBB3 significantly inhibited the invasion of HCT116 and SW480 cells *in vitro* (Figure 4B).

Discussion

The low expression of miR-143, which has a close correlation with the development, progression, metastasis and prognosis of cancer, has been frequently reported in different type of cancers[13-15]. However, very little was reported about miR-143 expression in CRC and its correlation with the clinicopathologic features of these patients. To address these issues, miR-143 expression levels and the clinicopathologic characteristics of 80 patients with colorectal cancer were examined. There was a significant association between miR-143 expression and lymphatic metastasis. However, no relationship was found between miR-143 expression and gender, tumor size, differentiation grade or pathologic stage suggesting that the low expression of miR-143 is significantly associated with CRC progression, especially for cancer metastasis.

The occurrence of invasion and metastasis after operation is the leading cause of poor outcome for CRC patients[16,17]. It is well known that miRNAs have been implicated in the regulation of a wide range of biological processes, including cell proliferation, invasion, differentiation, etc[18-20]. It was reported that the miR-143 expression was significantly down-regulated in different types of cancer, including esophageal cancer[13], prostate

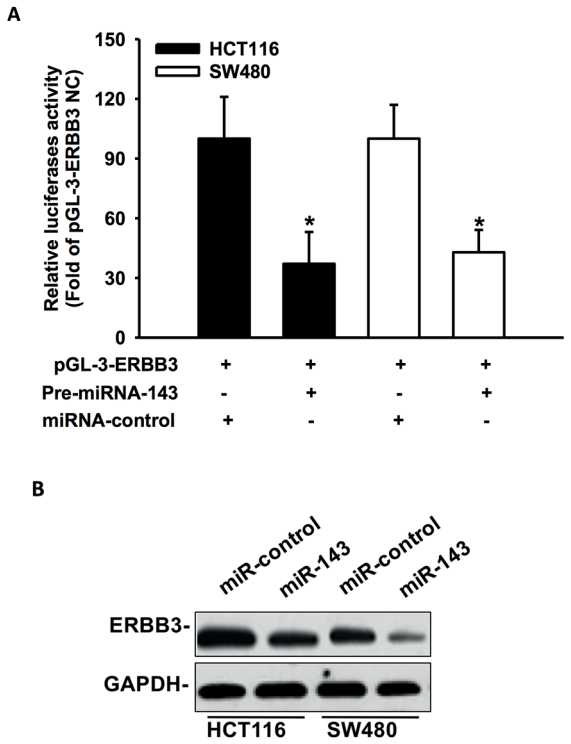

Figure 3. Effects of miR-143 on ERBB3 expression in HCT116 and SW480 cell lines. ***(A)*** Luciferase reporter assay was used to confirm the direct interaction between miR-143 and target ERBB3. ***(B)*** Overexpression of miR-143 in HCT116 and SW480 cells decreased the protein levels of ERBB3 determined by Western blot.

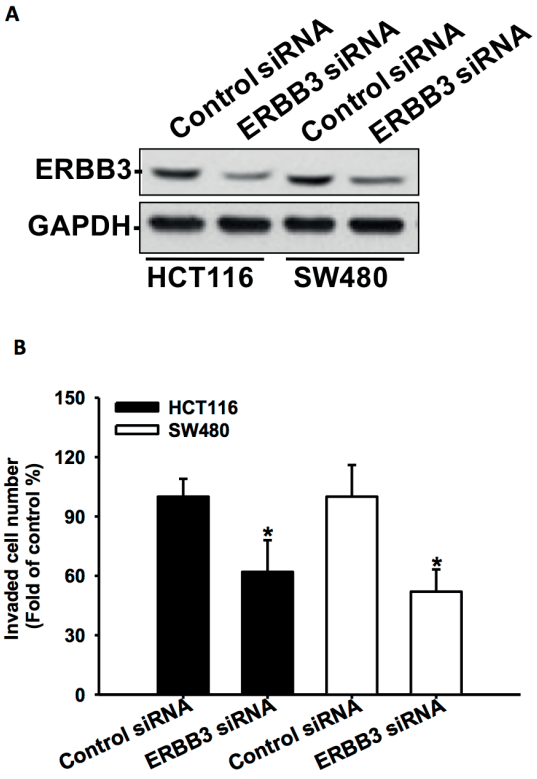

Figure 4. Suppression of ERBB3 by siRNA inhibited CRC cells invasion. ***(A)*** The protein levels of ERBB3 were detected by Western blot after transfected with ERBB3 siRNA and negative control. ***(B)*** ERBB3 siRNA significantly inhibited CRC cells invasion determined by transwell assay. Data were represented as a percentage of cell number relative to the control (100%) from 3 independent experiments. *Different ($p < 0.05$) from control.

cancer[14] and breast cancer[15]. These results were in agreement with our finding that miR-143 expression was down-regulated in colorectal cancer. Notably, we found that overexpressed miR-143 significantly suppressed colorectal cancer cell invasion, further suggested that miR-143 may play important role in the colorectal cancer metastasis and progression. Similarly, couple of studies also demonstrated that miR-143 positively suppressed cancer cells invasion in ovarian[21], breast[22], lung cancers[23].

In this work, ERBB3 was found and validated to be a target of miR-143 in CRC. ERBB3 is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases[24,25], which is deeply involved in the regulation of migration and invasion of cancer cells[26,27]. ERBB3 was frequently observed to be significantly increased in many different cancer types such as breast cancer[28], ovarian cancer[29] and lung cancer[30]. Recent importantly report documented that ERBB3 is a direct target for miR-143[31]. MiR-143 could repress the expression of ERBB3, which in turn inhibited cell migration and invasion in human breast cancer cells[31]. Consistent with this founding, our paper demonstrated that overexpression of miR-143 greatly silenced ERBB3 expression, subsequently impaired cell invasion in CRC. All these results documented that down-regulated miR-143 expression highly correlated with mobility and tumorigenicity of CRC probably by targeting ERBB3.

Conclusions

We proved that miR-143 was markedly down-regulated in human CRC tissues. Increasing the expression of miR-143 may lead to CRC cell invasion suppression through targeting ERBB3 pathway. Though there is still much to learn about the role of miR-143 in CRC metastasis and progression, miR-143 provides us with a new potential target for CRC treatment.

Conflicts of interest

The authors declare no conflicts of interest.

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