MicroRNA-144 suppresses aggressive phenotypes of tumor cells by targeting ANO1 in colorectal cancer

YASU JIANG[1*], YUNHUI CAI[2*], WEIWEI SHAO[2*], FENG LI[3], ZONGYU GUAN[4], YI ZHOU[4], CHONG TANG[1] and SHICHUN FENG[1]

[1]Department of General Surgery, The Second Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001; [2]Department of General Surgery, The Fourth Affiliated Hospital of Nantong University, Yancheng, Jiangsu 224006; [3]Department of Gastroenterology, Affiliated Hospital of Nantong University; [4]Medical College of Nantong University, Nantong, Jiangsu 226001, P.R. China

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Abstract. The aim of the present study was to research the mechanism of action of microRNA-144 (miR-144) in colorectal cancer (CRC) and its role in tumor progression. It was demonstrated that miR-144 was downregulated and anoctamin 1 (ANO1) expression was upregulated in CRC. The expression of ANO1 was negatively associated with that of miR-144 in CRC. The present study indicated that upregulated expression of ANO1 was associated with poor differentiation and advanced tumor-node-metastasis stage. It was verified that upregulation of ANO1 expression activated the epidermal growth factor receptor/extracellular signal-regulated kinase signaling pathway. It was also demonstrated that miR-144 exerts strong tumor-inhibiting effects by targeting ANO1. Therefore, miR-144 may have potential as a prognostic marker or therapeutic target for CRC.

Introduction

It has been well established that colorectal cancer (CRC) is one of the most common types of cancer. Among all types of malignant tumor, the morbidity and mortality rates of CRC are ranked third overall (1). In China, the incidence of CRC has been increasing steadily by ≤4.2% per year, and it is now the second most common cause of cancer-associated mortality (2). The majority of patients with CRC undergo surgical resection; however, recurrence occurs in 20-25% patients following a 'curative' operation (3). Multiple gene alterations, including the inactivation of tumor suppressor genes and the activation of oncogenes, are associated with the progression of the normal colonic epithelium into adenoma and malignant adenocarcinoma (4). However, there is limited knowledge of the molecular alterations that occur during the metastasis of CRC (5,6). Therefore, identification of the relevant genes and their molecular pathways is required to provide novel targets for the treatment of metastatic CRC. In recent years, microRNAs (miRNAs) have been demonstrated to be involved in the development of cancer.

Anoctamin 1 (ANO1) is located on human chromosome 11q13 and contains 26 exons, and encodes a 960-amino acid protein with 8 transmembrane domains. ANO1 is also referred to as TMEM16A, ORAOV2, TAOS2, DOG1 or FLJ10261. It is associated with the activity of calcium-dependent chloride channels expressed on the plasma membranes of secretory epithelia, smooth muscles and sensory neurons (7-12). ANO1 is also involved in a variety of biological functions, including cell proliferation, movement and attachment (13,14). In a large subset of head and neck squamous cell carcinoma cases, ANO1 is amplified and highly expressed (13), and the same is true for other types of cancer, including breast cancer (15), prostate carcinoma (14), glioblastoma (16), gastrointestinal stromal tumor (17) and esophageal squamous cell carcinoma (18). Certain studies have suggested that ANO1 may be a diagnostic and prognostic biomarker and a therapeutic target for various types of cancer (14,19). However, in human malignancies, the clinical implications of ANO1 remain to be elucidated.

miRNAs are a family of small noncoding RNA molecules, 21-25 nucleotides long. By targeting mRNAs, miRNAs are able to control the expression of ~30% of protein-coding genes by inhibiting their translation (20,21). miR-144 is enriched in the brain, and exists in normal and malignant hematopoietic cells (22). Previous reports have demonstrated that miR-144 serves a role in the regulation of cell proliferation (23) and apoptosis (24), in addition to the progression of various types of human cancer, including nasopharyngeal carcinoma (25), CRC (26), follicular thyroid carcinoma (19) and pancreatic cancer (27). In addition, miR-144 has been demonstrated to

Correspondence to: Mr. Shichun Feng or Mr. Chong Tang, Department of General Surgery, The Second Affiliated Hospital of Nantong University, 6 Nantong Haier Lane Road, Nantong, Jiangsu 226001, P.R. China
E-mail: fengshichungs@sina.com
E-mail: 15051273108@163.com

*Contributed equally

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target signaling pathways, including the protein kinase C, Wnt/β-catenin and phosphatase and tensin homolog (PTEN) pathways (28).

miRanda and TargetScan were first used to identify miR-144 as an ANO1-targeting miRNA in CRC. Research is limited regarding the function of miR-144 in CRC by targeting ANO1. Therefore, the present study aimed to analyze miR-144 expression by targeting ANO1 in human CRC tissue specimens.

Materials and methods

Materials. A total of 122 tissue samples from patients with CRC between April 2005 and April 2009 were collected and formalin-fixed for 48 h at room temperature, and paraffin-embedded. Each patient underwent surgical resection at The Second Affiliated Hospital of Nantong University (Nantong, China). Patient characteristics are presented in Table I. No patients had received prior radiotherapy or chemotherapy. CRC tumor and adjacent non-cancerous tissue samples were obtained randomly from 26 patients with CRC. The fresh samples were frozen at -80˚C until required for western blot analysis. Clinicopathological data, including sex, age, tumor size, tumor-node-metastasis (TNM) stage and lymph node involvement, were retrospectively analyzed. Staging and grading of the CRC were performed according to the World Health Organization classification and the Union for International Cancer Control (29). Survival time was defined as the time between surgery and the time of the last follow-up day, or patient mortality. The present study was conducted with the approval of the institutional ethics board of The Second Affiliated Hospital of Nantong University (no. 2006-K013) and the patients with CRC included in the present study had provided written informed consent.

Immunohistochemistry. Immunohistochemical staining was performed on 4-μm thick sections. Tissues were de-waxed in xylene and rehydrated in alcohol in a descending series, and endogenous peroxidase activity was suppressed using 3% hydrogen peroxide for 10 min at room temperature. Antigen retrieval was performed by treatment at 100˚C in 0.01 mol/l sodium citrate buffer (pH 6.0) for 10 min. The sections were then rinsed with PBS twice for 5 min, and non-specific binding was blocked using 10% normal goat serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at room temperature. Sections were incubated at 4˚C overnight with a polyclonal rabbit anti-human ANO1 antibody (1:100; cat. no. sc-377115; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in PBS. Following three 10-min washes in PBS, the sections were incubated with IRDye® 800CW-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. 925-32211; Rockland Immunochemicals, Inc., Limerick, PA, USA) for 1 h at 37˚C. Following washing in PBS three times, diaminobenzidine solution was used to develop the visualization signal. Following hematoxylin counterstaining at room temperature for 5 min, the sections were dehydrated and mounted. All slides were examined using a light microscope (Leica Microsystems GmbH, Wetzlar, Germany) and the results of the immunohistochemical staining were assessed separately by three investigators, considering staining frequency as follows: No staining, 0; 1-25% of cells stained, 1; 25-50% of cells stained, 2; 51-75% of cells stained, 3; and >75% of cells stained, 4. Staining intensity was rated on a scale of 0-12: 0, negative staining (-); 1-4, weak staining (+); 5-8, moderate staining (++); and 9-12, strong staining (+++).

Western blotting. Radioimmunoprecipitation assay lysis buffer containing protease inhibitors (Promega Corporation, Madison, WI, USA) was used to extract the total protein from the tissues. Equal amounts (30 μg) of protein were examined using the DC protein assay method (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and separated by SDS-PAGE on a 10% gel, and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked in 5% non-fat dry milk in Tween-20 TBS (TBST) buffer (50 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween-20; pH 7.4) at room temperature for 1 h. The membrane was incubated with a polyclonal rabbit anti-human ANO1 antibody (cat. no. sc-377115; 1:500; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. The other specific primary antibodies used against each protein in the immunoblotting were as follows: Anti-EGFR (cat. no. sc-71034), anti-p-EGFR (cat. no. sc-81487), anti-ERK1/2 (cat. no. sc-81457) and anti-p-ERK1/2 (cat. no. sc-7976) and anti-β-actin (cat. no. sc-8432) (all 1:1,000; Santa Cruz Biotechnology, Inc., USA). Following three washes in TBST for 5 min, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (cat. no. A6154; 1:1,000; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 2 h. An Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) was used to scan the membrane, and PDQuest software (version, 7.2.0; Bio-Rad Laboratories, Inc.) was used to analyze the protein bands.

Cell culture and transfection. The normal intestinal epithelial cell line FHC, and the human colorectal carcinoma cell lines Caco-2, SW620, HCT116, SW480 and LoVo were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), except SW480 and SW620 cells, which were cultured in Leibovitz's L-15 Medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS, at 37˚C in a 5% CO_2 atmosphere in a humidified incubator. miR-144 mimics (miR-144), miR-144 negative control (miR-NC) and the miR-144 inhibitor were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences were as follows: miR-144 mimic sense, 5'-UACAGUAUAGAUGAUGUACU-3'; miR-NC sense, and 5'-UUCUCCGAACGUGUCACGUTT-3'; miR-144 inhibitor, 5'-AGUACAUCAUCUAUACUGUA-3'. The sequences used for ANO1 siRNA were as follows: ANO1 siRNA forward, 5'-UUUAUUUAGAUGAAUGUCCAG-3' and reverse, 5'-GGACAUUCAUCUAAAUAAAUU-3'; control siRNA forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. SW480 cells ($1x10^6$) were cultured in 6-well plates, and the cell density reached 40-60% confluence after 24 h. Oligonucleotides (50 nmol) were transfected into cells using Lipofectamine® 2000 (Thermo Fisher

Table I. Association between ANO1 expression in colorectal cancer tissues and clinical parameters.

Clinical parameters	No. of patients (n=122)	ANO1 expression		χ^2	P-value
		Low level (n=44)	High level (n=78)		
Sex				3.303	0.069
Male	66	19	47		
Female	56	25	31		
Age, years				0.458	0.499
≤50	35	11	24		
>50	87	33	54		
Tumor size, cm				0.120	0.729
≤5	64	24	40		
>5	58	20	38		
Tumor location				0.647	0.421
Colon	69	27	42		
Rectum	53	17	36		
Gross type				1.473	0.479
Massive	48	16	32		
Ulcerative	53	18	35		
Infiltrating type	21	10	11		
Histological differentiation				26.202	<0.001[a]
Well/moderate	91	21	70		
Poor	31	23	8		
Lymph node metastasis				1.396	0.237
Negative	75	24	51		
Positive	47	20	27		
Invasive depth				0.054	0.817
T1/T2	51	19	32		
T3/T4	71	25	46		
TNM stage				8.816	0.003[a]
I/II	67	32	35		
III/IV	55	12	43		

Statistical analyses were performed using the Pearson χ^2 test. [a]P<0.05 was considered to indicate a statistically significant difference. ANO1, anoctamin 1; TNM, tumor-node-metastasis.

Scientific, Inc.), according to the manufacturer's protocol. Following transfection for 48 h, the cells were used for further cellular function analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissue samples and cells using the RNeasy RNA Mini kit (Qiagen GmbH, Hilden, Germany). To detect the expression of mature miR-144, cDNAs were synthesized using a TaqMan MicroRNA Reverse Transcription kit at a reaction temperature of 42˚C (Applied Biosystems; Thermo Fisher Scientific, Inc.) and quantified by qPCR using a TaqMan human microRNA assay kit (Qiagen), according to the manufacturer's protocol. qPCR was performed for amplification under the following thermocycling conditions: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. The relative expression ratio of miR-144 was presented as the fold-change normalized to a U6 endogenous reference in the normal cell line and was calculated by the $2^{-\Delta\Delta Ct}$ method (30).

Luciferase reporter assay. The ANO1 3' untranslated regions (UTRs) containing the wild-type (WT) or mutant (MT) miR-144 binding site were amplified and cloned into a pGL3-basic vector (Promega Corporation), and termed ANO1-WT-3'UTR and ANO1-MT-3'UTR, respectively. The cells were seeded into 24-well plates and co-transfected with 50 nmol/l pcDNA-miR-144 or the negative control (NC) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). *Renilla* luciferase activity was used as an internal control. Following incubation for 24 h, the luciferase assay was tested using a dual-luciferase reporter assay system (Promega Corporation), according to the manufacturer's protocol.

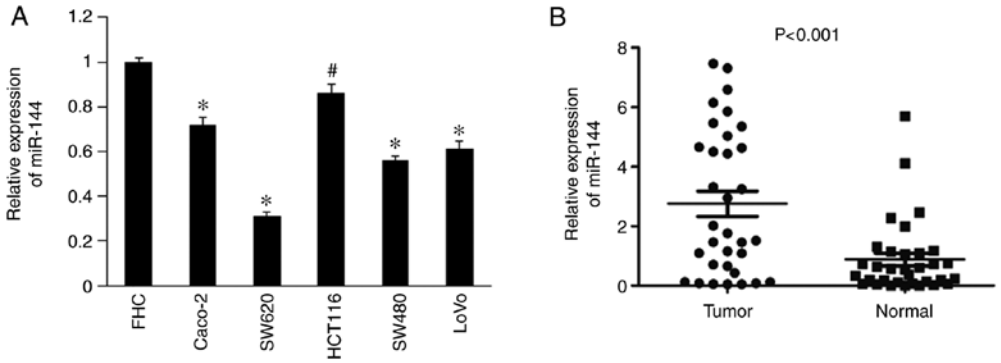

Figure 1. miR-144 expression is significantly reduced in colorectal cancer cell lines compared with normal cells. (A) Relative expression levels of miR-144 different cell lines. #P<0.05, *P<0.01 vs. FHC. (B) Relative expression levels of miR-144 in normal tissues (n=33) and CRC tissues (P<0.001). miR, microRNA.

Migration and invasion assays. Transwell chamber plates (24-well) were used to evaluate cell migration and invasion. For the migration assay, $5x10^4$ cells were suspended in 200 ml serum-free DMEM and seeded into the upper chamber of each insert. Next, 800 μl DMEM containing 10% FBS was added to the lower chambers. For the invasion assay, 40-80 μl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) diluted with DMEM was used to coat the chambers and incubated for 2-4 h at 37˚C. A total of $1x10^5$ cells were suspended in 200 μl DMEM and seeded in the upper chambers, and 800 μl DMEM containing 10% FBS was added to the lower chambers. The plates were incubated at room temperature for 24 h, and the migratory and invasive cells were fixed and stained in a 20% methanol and 0.2% crystal violet solution for 30 min at 37˚C. The numbers of migratory or invasive cells were counted in three fields in each well under a light microscope at x200 magnification.

Statistical analysis. The SPSS software package (version 17.0; SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. The miRNA target predicting algorithms TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do) were used to predict potential targets of miR-144. The paired data were analyzed using a paired Student's t-test, while comparisons between unpaired groups were performed using an unpaired Student's t-test. The association between ANO1 expression and clinicopathological features was analyzed using χ^2 test. In addition, one-way analysis of variance with Tukey's post hoc test was used to analyze the data differences between groups. The Kaplan-Meier method was used to calculate overall survival curves, which were analyzed using the log-rank test. The Cox proportional hazards regression model was used to perform univariate and multivariate analysis of several prognostic factors. The results are representative of at least three independent experiments. Data are presented as the mean ± standard deviation from three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of miR-144 in CRC cell lines and tissues. According to the RT-qPCR results, miR-144 levels were significantly lower in the CRC cell lines, Caco-2, SW620, HCT116, SW480 and LoVo, compared with normal FHC cells (P<0.05; Fig. 1A). As presented in Fig. 1B, it was demonstrated that miR-144 expression was significantly decreased in tumor tissues compared with paracancerous tissues (n=33; P<0.001).

miR-144 inhibits cell migration and invasion in vitro. The transfection efficiency was confirmed by flow cytometry. Flow cytometric analysis confirmed that the transfection achieved ~90% efficiency (Fig. 2A). The relative miR-144 expression levels were determined by RT-qPCR following miR-144 mimic overexpression in SW480 cells. miR-144 mRNA expression levels were decreased following miR-144 inhibition in SW480 cells (Fig. 2B). To determine the effects of miR-144 on the migration and invasion of CRC cells, SW480 cells transfected with either miR-144 mimic or miR-144 inhibitor were subjected to Transwell assays. The migratory and invasive abilities of SW480 cells transfected with miR-144 mimic were reduced compared with the miR-NC group (Fig. 2C; P<0.05). Downregulated expression of miR-144 via transfection with miR-144 inhibitor promoted cell migration and invasion in SW480 cells compared with the miR-NC group (Fig. 3; P<0.05).

miR-144 targets ANO1 via binding to its 3'UTR. To elucidate the potential molecular mechanism of miR-144 in CRC progression, putative target genes of miR-144 were identified using the TargetScan and miRanda databases. It was revealed that the 3'UTR of ANO1 mRNA contains potential binding sites for miR-144 (Fig. 4A). A luciferase activity assay was used to assess whether ANO1 is a direct target of miR-144. SW480 cells were co-transfected with ANO1-WT-3'UTR or ANO1-MT-3'UTR, pcDNA-miR-144 or NC. As demonstrated in Fig. 4B, miR-144 decreased the relative luciferase activity of ANO1-WT-3'UTR in SW480 cells compared with miR-NC (P<0.05), but not in cells transfected with ANO1-MT-3'UTR. RT-qPCR and western blot analysis also demonstrated that the mRNA (P<0.05; Fig. 4C) and protein (P<0.05; Fig. 4D) expression levels of ANO1 were significantly decreased following miR-144 overexpression in SW480 cells, compared with cells transfected with miR-NC. It was also observed that the mRNA (P<0.05; Fig. 4E) and protein (P<0.05; Fig. 4F) expression of ANO1 was increased in SW480 cells transfected with miR-144 inhibitor, compared with cells transfected with miR-NC, by RT-qPCR and western blot analysis.

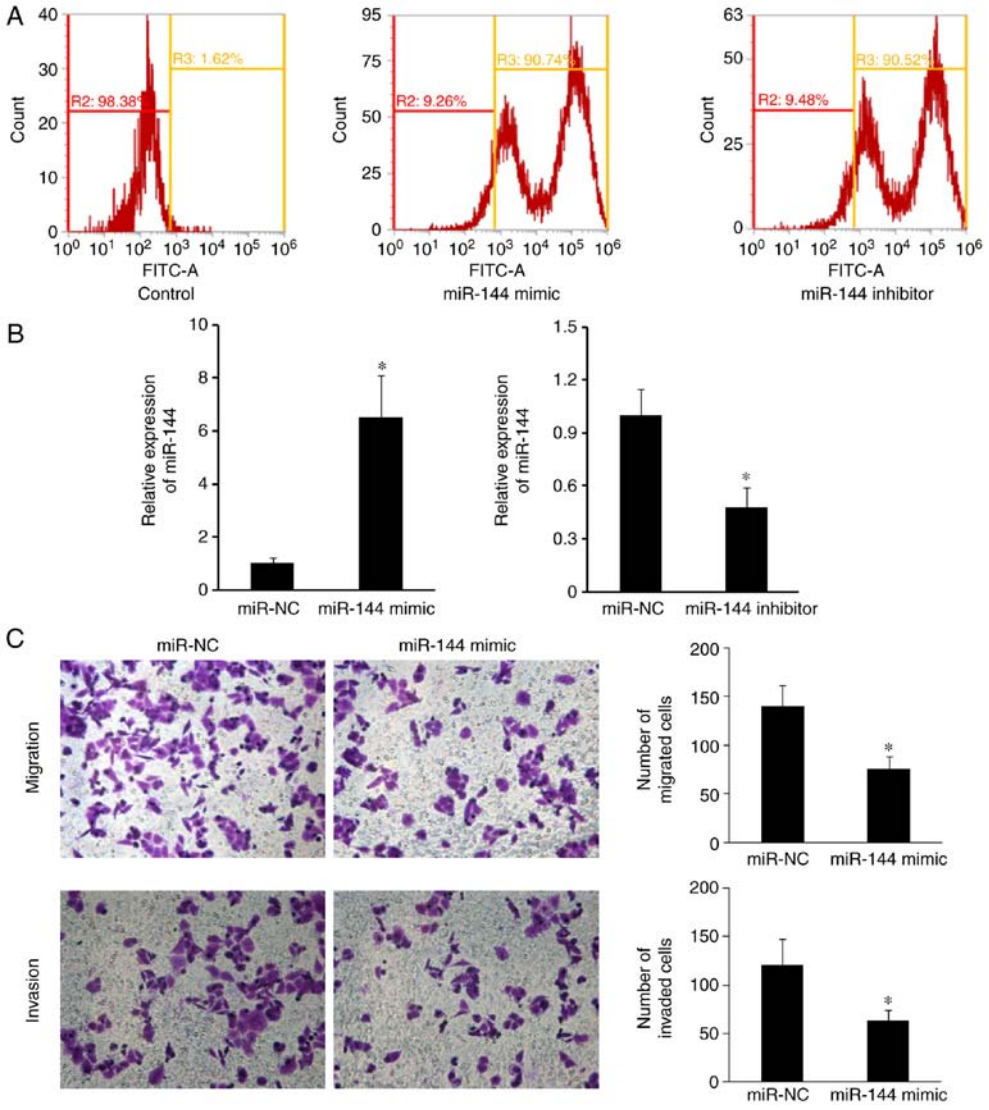

Figure 2. Ectopic miR-144 expression suppresses colorectal carcinoma cell migration and invasion. (A) The transfection efficiency was detected by flow cytometry following transfection for 24 h. The transfection efficiency reached ~90%, which indicated that the cells had been successfully transfected with miR-144 mimic and inhibitor using Lipofectamine® 2000. (B) Reverse transcription-quantitative polymerase chain reaction analysis was performed to determine the relative expression of miR-144 in SW480 cells following transfection with the miR-144 mimic or inhibitor. The miR-NC group represents SW480 cells transfected with the scramble miRNA. (C) Ectopic miR-144 expression significantly inhibited cell migration and invasion. Original magnification, x200. *P<0.05 vs. miR-NC. miR, microRNA; NC, negative control; FITC, fluorescein isothiocyanate.

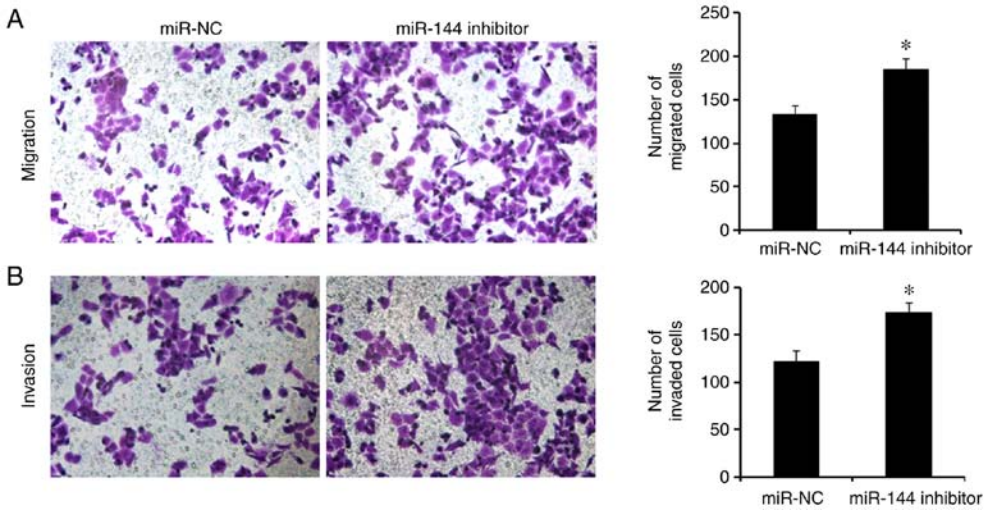

Figure 3. Blocking of endogenous miR-144 promotes colorectal carcinoma migration and invasion. (A) Knockdown of miR-144 significantly increased cell migration (B) Knockdown of miR-144 significantly increased cell invasion. Original magnification, x200. *P<0.05 vs. miR-NC. miR, microRNA; NC, negative control.

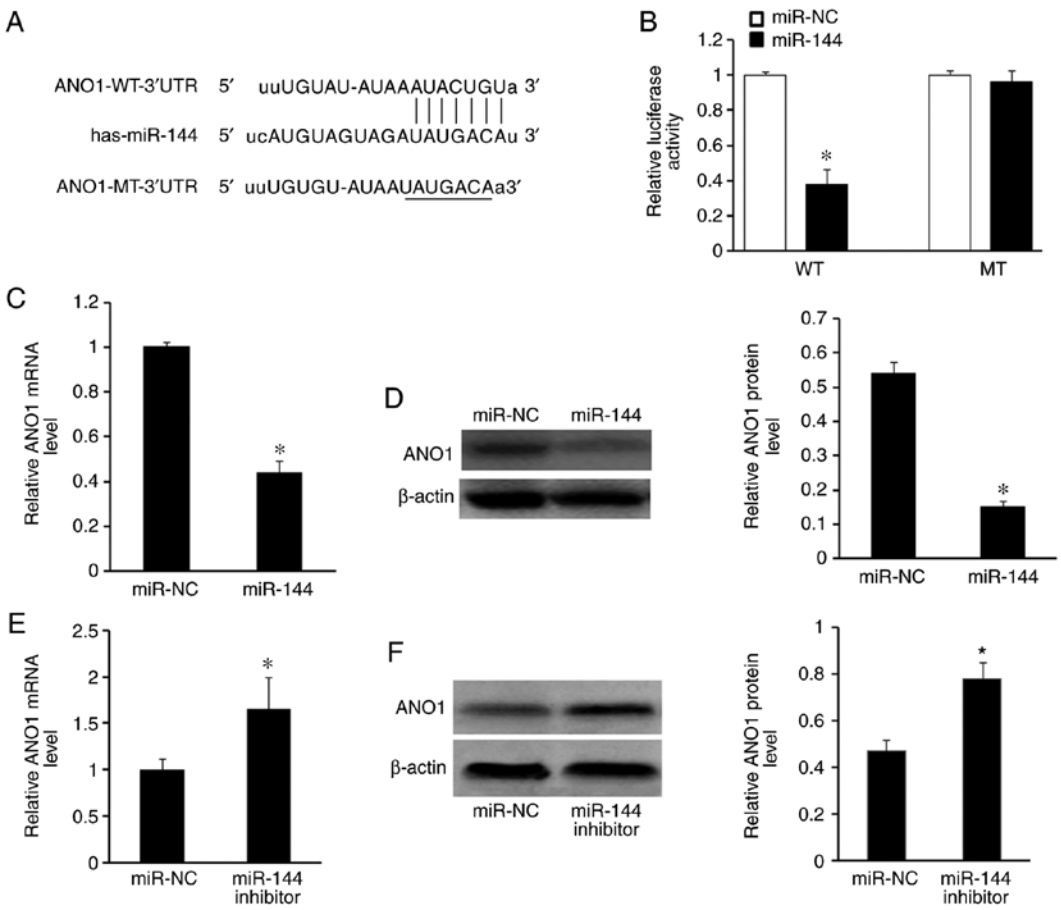

Figure 4. ANO1 acts as a target of miR-144 in CRC cells. (A) The 3'UTR of ANO1 mRNA contains binding sequences for miR-144. (B) Cotransfection of pGL3-WT-ANO1 and pcDNA-miR-144 reduced the levels of luciferase activity in SW480 cells, but cotransfection of pGL3-MT-ANO1 and pcDNA-miR-144 did not. (C) The expression of ectopic miR-144 reduced the mRNA expression level of ANO1 in SW480 cells. (D) The expression of ectopic miR-144 reduced the protein expression level of ANO1 protein in CRC cells. (E) miR-144 inhibitor upregulated the mRNA expression level of ANO1 in SW480 cells. (F) The expression of miR-144 inhibitor increased the protein expression level of ANO1 in CRC cells. *P<0.05 vs. respective miR-NC group. UTR, untranslated region; ANO1, anoctamin 1; miR, microRNA; NC, negative control; WT, wild-type; MT, mutant; CRC, colorectal cancer.

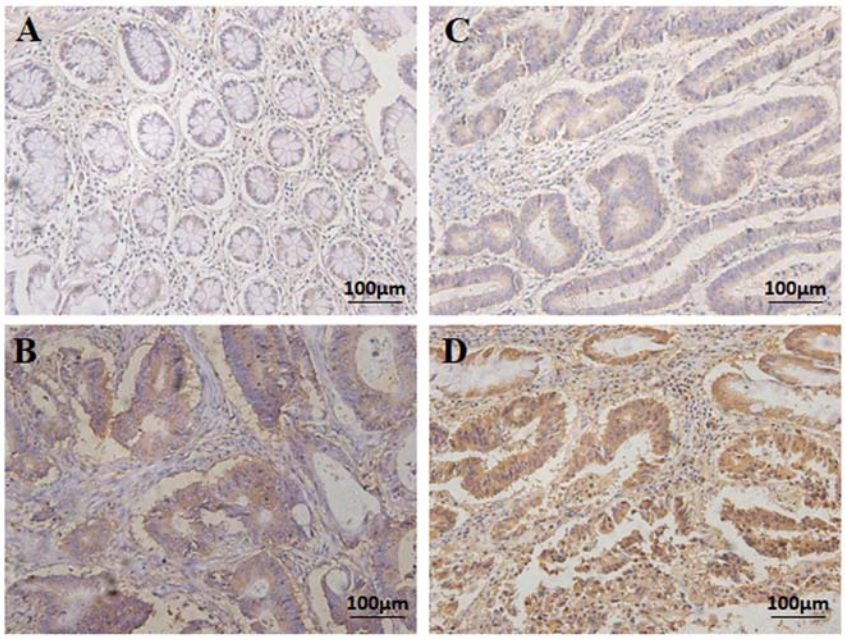

Figure 5. Expression of ANO1 protein in CRC and paired adjacent non-tumor tissues detected by immunohistochemical staining. (A) No staining of ANO1 was detected in adjacent normal tissues. (B) Weak staining of ANO1 was detected in well-differentiated CRC tissues. (C) Moderate staining of ANO1 was detected in moderately differentiated CRC tissues. (D) Strong staining of ANO1 was detected in poorly-differentiated CRC tissues. Magnification, x200. ANO1, anoctamin 1; CRC, colorectal cancer.

Table II. Cox proportional hazards model analysis of prognostic factors.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
ANO1 expression (high vs. low)	1.06	0.85-1.17	0.018[a]	1.38	0.84-1.94	0.032[a]
TNM stage (III/IV vs. I/II)	0.85	0.73-1.05	0.776	0.92	0.56-1.68	0.089
Lymph node metastasis (positive vs. negative)	0.89	0.68-1.16	0.042[a]	0.54	0.28-1.36	0.970
Invasive depth (T3/T4 vs. T1/T2)	0.68	0.43-0.85	0.184	0.48	0.26-1.06	0.067
Histology differentiation (poor vs. well/moderate)	1.34	0.83-1.87	0.005[a]	1.09	0.23-2.58	0.003[a]
Tumor location (rectum vs. colon)	0.63	0.52-1.13	0.325	0.49	0.28-1.17	0.092
Tumor size (>5 cm vs. ≤5 cm)	0.07	0.41-1.40	0.093	0.67	0.32-1.48	0.296

Statistical analyses were performed by the Cox regression analysis. [a]P<0.05 was considered to indicate a statistically significant difference. HR, hazard ratio; TNM, tumor-node-metastasis; CI, confidence interval; ANO1, anoctamin 1.

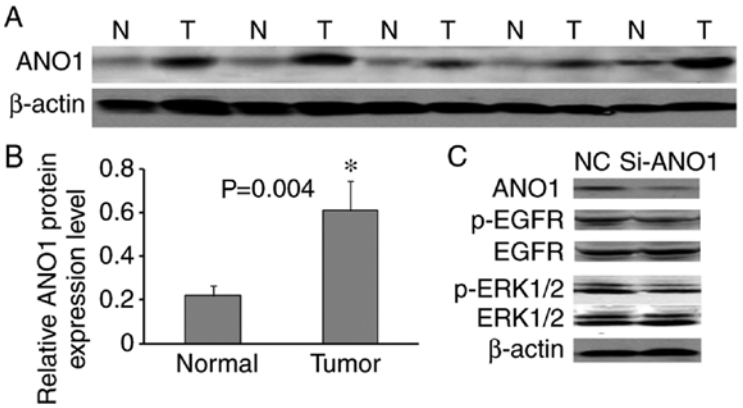

Figure 6. Expression of ANO1 was increased in CRC tissues compared with normal tissues. (A) Western blot analysis of five representative paired tissue samples. (B) Western blotting results were normalized to β-actin expression in the same samples. The protein expression level of ANO1 in CRC was significantly higher compared with that in adjacent normal tissue. (C) The protein expression levels of SW480/NC and SW480/Si-ANO1 were analyzed by western blotting. T, CRC tissue; N, adjacent normal tissue; CRC, colorectal cancer; ANO1, anoctamin 1; EGFR, epidermal growth factor receptor; p, phosphorylated; ERK, extracellular signal-regulated kinase; NC, negative control; Si, small interfering. *P<0.05.

Association between the expression of ANO1 and various clinicopathological characteristics. According to the 122 paraffin-embedded colorectal tissue blocks evaluated by immunohistochemistry, it was demonstrated that ANO1 was expressed in the CRC tissues samples. A total of 78/122 (63.93%) cases exhibited high ANO1 expression (ANO1 ++ or ANO1 +++), whereas 44/122 (36.07%) exhibited low ANO1 expression (ANO1- or ANO1 +) (Fig. 5; Table I). The adjacent noncancerous colorectal tissues exhibited no ANO1 staining (Fig. 5).

According to the χ^2 analysis, the overexpression of ANO1 was associated with clinicopathological parameters, including histological grade (P<0.001) and TNM stage (P=0.003). However, it was not associated with sex, age, tumor location or tumor size (P>0.05; Table I).

Upregulation of ANO1 in CRC tissue samples. Western blotting was used to analyze the protein expression of ANO1 in 26 randomly selected CRC and matched non-tumor tissues. The results of five representative cases are presented in Fig. 6A. β-actin was used to normalize ANO1 protein expression. Compared with adjacent non-tumor tissues in CRC patients, ANO1 protein was upregulated in 69.2% of CRC tissues, and in 26 CRC tissues, the average ANO1 protein expression level was significantly higher compared with that in the adjacent non-tumor colorectal tissues (P=0.004; Fig. 6B).

The total and phosphorylated levels of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK)1/2 were also investigated. Downregulation of ANO1 suppressed the phosphorylation of EGFR and ERK1/2, but had no effect on the total EGFR and ERK1/2 protein expression levels (Fig. 6C).

miR-144 suppresses CRC cell migration and invasion by downregulating ANO1. To investigate whether the effects caused by blocking miR-144 may be abolished by knockdown of ANO1, SW480 cells were co-transfected with miR-144 inhibitor and si-ANO1. Western blotting revealed that the induction of ANO1 expression caused by miR-144 inhibitor may be partly rescued by the suppression of ANO1 expression using si-ANO1 (Fig. 7A). The Transwell assays indicated that inhibition of ANO1 abrogated the miR-144 inhibitor-mediated

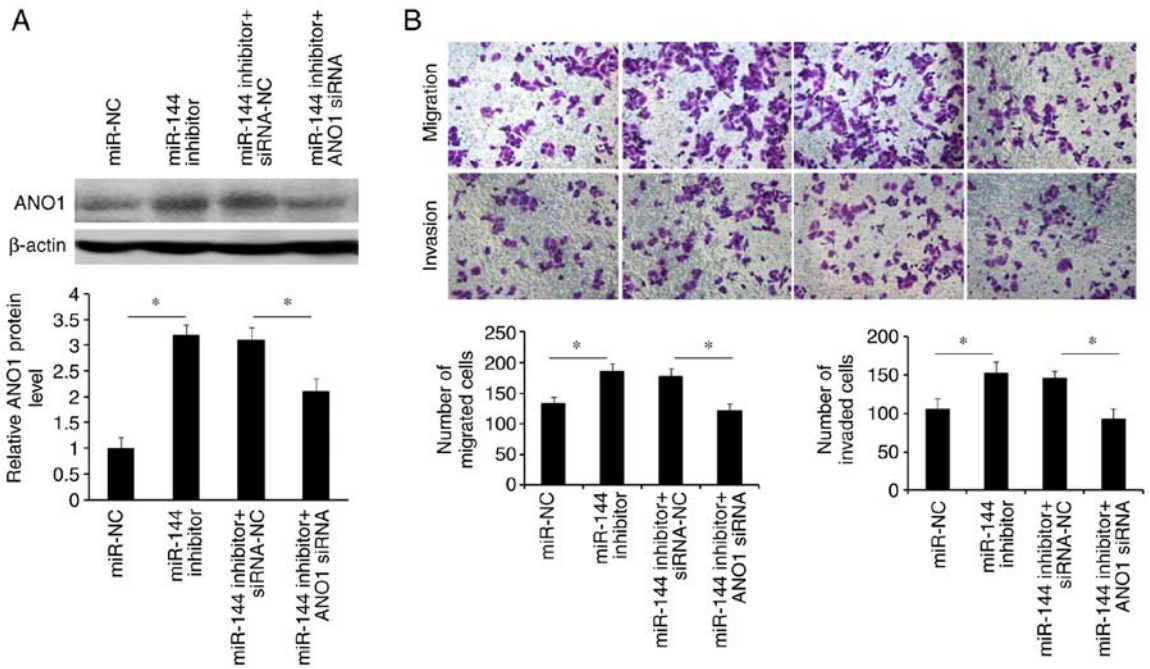

Figure 7. miR-144-suppressed colorectal cancer cell migration and invasion was mediated by downregulation of ANO1 expression. (A) The induction of ANO1 expression caused by miR-144 inhibition may be partly rescued by the suppression of ANO1 expression using si-ANO1. (B) Inhibition of ANO1 reduced miR-144 inhibitor-mediated SW480 cell migration and invasion. Original magnification, x200. *P<0.05. miR, microRNA; ANO1, anoctamin 1; si, small interfering; NC, negative control.

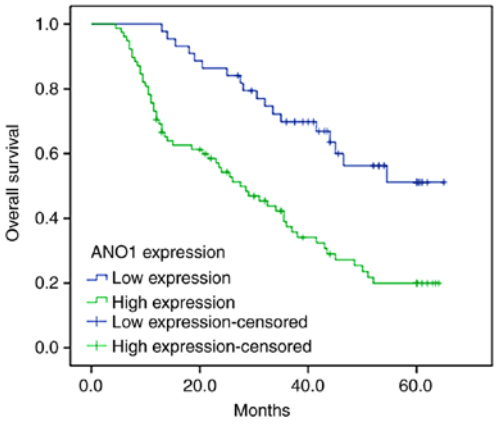

Figure 8. Prognostic significance of ANO1 expression in the patients with CRC. Kaplan-Meier curves for overall survival in patients with CRC with low and high levels of ANO1 expression (χ^2=15.76; P<0.001). CRC, colorectal cancer; ANO1, anoctamin 1.

migration and invasion of SW480 cells (Fig. 7B). These results suggested that miR-144-suppressed CRC cell migration and invasion was mediated by downregulation of ANO1.

Association between ANO1 expression and patient survival. Survival analysis of the high and low ANO1 expression groups was performed to evaluate the prognostic value of ANO1 in patients with CRC. Compared with the high expression group, patients exhibiting low ANO1 expression had a longer overall survival time (P<0.001; Fig. 8). According to the multivariate Cox regression analysis, it was demonstrated that the expression of ANO1 and differentiation were significantly associated with the overall survival time of patients with CRC (Table II).

Discussion

Colorectal cancer (CRC) has one of the highest mortality rates among malignant tumors worldwide. According to the World Health Organization's International Agency for Research on Cancer, CRC is the second most common type of malignant tumor in females and the third most common in males (31). Multiple genetic alterations are involved in the progression of the normal colonic epithelium into adenoma and subsequent malignant adenocarcinoma (32). However, understanding of the molecular alterations in the metastasis of CRC is limited.

ANO1 mediates trans-epithelial ion transport. It serves an important role in regulating airway fluid secretion, gut motility, secretory functions of exocrine glands, renal function, smooth muscle contraction and nociception (10,12,33). Certain diseases, including cystic fibrosis, asthma, gastroparesis, hypertension, rotavirus-induced diarrhea and polycystic kidney disease, are associated with ANO1 dysfunction (34-38). In a large subset of head and neck squamous cell carcinoma cases, ANO1 is amplified and highly expressed (13), which is also the case for other types of cancer including breast cancer (15), prostate carcinoma (15), glioblastoma (16), gastrointestinal stromal tumors (17), and esophageal squamous cell carcinoma (18).

Previous studies have demonstrated that miR-144 is associated with the response to mood stabilizer treatment (28), stress responses (39) and aging diseases (40). In various types of cancer, aberrant expression of miR-144 has been demonstrated. It has been reported that downregulation of miR-144 may be associated with poor prognosis in patients with CRC due to activation of the mTOR signaling pathway (26). It has also been revealed that miR-144 may regulate proliferation by targeting enhancer of zeste homolog 2 in bladder cancer cells (41). Other observations have suggested that miR-144 activates RAC-α

serine/threonine-protein kinase signaling via downregulation of PTEN in the tumorigenesis and tumor progression of breast cancer (42). In the present study, miR-144 was downregulated in CRC and it significantly inhibited cell migration and invasion *in vitro*. Downregulation of miR-144 via transfection with miR-144 inhibitor significantly promoted CRC cell migration and invasion. In addition, miR-144 was involved in ANO1-mediated CRC cell migration and invasion. Furthermore, the data presented herein suggest that ANO1 serves consistent roles in the activation of the EGFR/ERK signaling pathway.

Due to the limited number of patients included in the study, the Cox proportional hazards regression model did not indicate a significant association between TNM stage and patient survival. Therefore, a future study may use a larger number of samples. Furthermore, the present study demonstrated that miR-144 serves a critical role in CRC carcinogenesis. It was identified that ANO1 is a direct functional target of miR-144. To confirm the results, the effect of ANO1 overexpression and silencing on the growth, migration and invasion capabilities of multiple CRC cell lines may be examined in future studies. It is essential to understand the molecular mechanism behind the miR-144-mediated regulation of ANO1 expression.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SF, CT and YC had responsibility for the design of the study. YJ, FL, ZG and YZ performed the experiments. YC, WS and FL analyzed the data. CT, YJ and WS wrote the manuscript. SF ZG and YZ revised the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was conducted with the approval of the institutional ethics board of The Second Affiliated Hospital of Nantong University and the patients included in the present study had provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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