MicroRNA-144 mediates chronic inflammation and tumorigenesis in colorectal cancer progression via regulating C-X-C motif chemokine ligand 11

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Abstract. Colorectal cancer (CRC) is one of the most common malignancies worldwide. The aim of the present study was to investigate the expression of microRNA-144 (miR-144) and C-X-C motif chemokine ligand 11 (CXCL11) in CRC and their association. Data from Gene Expression Omnibus (GEO) DataSets were analyzed to obtain the expression profile of CXCL11 in CRC. Subsequently, serum samples were collected from 65 subjects, including 39 patients with CRC and 26 controls; CRC and adjacent normal tissues were collected from all 39 CRC patients and the expression of CXCL11 was measured in these specimens. After searching for the potential regulator of CXCL11 through bioinformatics analysis, the levels of miR-144 in the clinical specimens were also detected. Finally, the regulatory association between miR-144 and CXCL11 was certified via the dual-luciferase reporter assay. Microarray data and bioinformatics analysis demonstrated that CXCL11 was significantly upregulated in CRC tissues and miR-144 was a potential regulator of CXCL11. In line with this finding, the expression of CXCL11 was significantly increased in the serum and tumor samples of patients with CRC, while that of miR-144 was downregulated. Dual-luciferase reporter assay revealed that miR-144 directly targets the 3'-untranslated region of CXCL11 mRNA to regulate its expression. These results demonstrated that enhanced CXCL11 expression in patients with CRC was associated with reduced miR-144 expression. The results of the present study may indicate a novel regulatory role of miR-144 in CRC through CXCL11 downregulation.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide (1). Over the last 25 years, the incidence and mortality of CRC in China have markedly increased (2). CRC is characterized by invasion and metastasis, and the 5-year survival rate of patients with distant metastasis is <10% (3).

Previous studies demonstrated that chronic inflammation is an important initiation event in CRC tumorigenesis (4). C-X-C motif chemokine ligand 11 (CXCL11), released by immune cells during inflammation, is a small cytokine that may contribute to progression of colonic tumorigenesis (5). CXCL11 may regulate the chemotaxis of cells through interaction with a subset of 7-transmembrane, G protein-coupled receptors (6). In CRC, CXCL11 induces infiltration by tumor-associated macrophages and is associated with poor patient prognosis (7). Colon carcinoma cells induce the migration of CXCR3-expressing cytotoxic T lymphocytes in a CXCL11-dependent manner (8). All evidence suggests that CXCL11 is one of the key cytokines interlinking inflammation and CRC.

MicroRNAs (miRNAs) are short non-coding RNAs that regulate the expression of their targets at the mRNA level (9). Accumulating evidence has demonstrated that the dysregulation of miRNAs is responsible for the pathogenesis of CRC (10). miR-144 is widely present in human tissues and body fluids, but its levels are abnormal under disease conditions (11). miR-144 is significantly downregulated in various tumor tissues, including CRC (12,13). Being a tumor suppressor, miR-144 upregulation may inhibit the proliferation, invasion and metastasis of cancer cells (14,15). However, an association between miR-144 and the CXCL11 signaling pathway has not been reported to date. The present study was undertaken to analyze the expression profile of CXCL11 in

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CRC from Gene Expression Omnibus (GEO) datasets. In addition, we investigated the expression of CXCL11 and miR-144 in the serum and tumor specimens of patients with CRC, in order to determine whether there is a regulatory association between miR-144 and CXCL11.

Materials and methods

Microarray data. Microarray dataset GDS4382, GDS4515, GDS3756 and GDS2947 were downloaded from the Gene Expression Omnibus database (GEO DataSets). GDS4382 (16), based on GPL570 platform, consisted of 17 CRC tumor tissues and 17 normal tissues; GDS4515 (17), based on GPL96 platform, included 34 microsatellite-unstable colorectal tumor samples and 15 normal colonic mucosa samples; 22 rectal tumor samples and 20 normal rectal tissue samples were selected from GDS3756 (18) based on GPL2986 platform; the array data of GDS2947 (19), based on GPL570 platform, included 32 adenoma samples and 32 normal mucosa samples. Under the same experimental conditions, tumor samples and normal samples were divided into two groups for screening. Data Analysis Tools in GEO DataSets was used for identifying differentially expressed genes (DEGs). The cut-off criterion was set as P<0.05 and value means difference >2+ fold.

Prediction of regulator for CXCL11. The prediction of regulator for CXCL11 was performed by miRWalk 1.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html), a database that not only documents miRNA binding sites within the complete sequence of a gene, but also combines this information with a comparison of binding sites resulting from other existing miRNA-target prediction programs (20). A total of 10 established miRNA-target prediction programs (Diana-microT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22 and TargetScan) were available in miRWalk. The potential targets of one miRNA are considered to be the genes that have been predicted by at least 5 programs (21).

Patients and samples. To determine the expression of genes or miRNAs in CRC tissues, tumor and surrounding normal tissue samples were collected from 39 patients with CRC (23 men and 16 women; age range, 25-65 years; median age, 45.3 years). All the patients had undergone radical surgery at the Department of General Surgery, West China Hospital, Sichuan University, between May 2010 and September 2011. Prior to surgery, none of the patients had received chemotherapy or radiotherapy. The excised tumors and adjacent tissues were stored in liquid nitrogen for molecular biology experiments. In addition, peripheral blood was collected from all 39 patients and 26 healthy control subjects (16 men and 10 women; age range, 23-66 years; median age, 44.8 years). The separation of serum was performed by centrifugation at 1,000 x g for 10 min at 4°C. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Tissue and blood samples were collected from consenting individuals according to the protocols approved by the Ethics Review Board at Sichuan University.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissue samples using E.Z.N.A.® Total DNA/RNA/Protein kit (Omega Bio-Tek, Inc., Norcross, GA, USA). Total RNA was extracted from serum samples using the GenElute™ Plasma/Serum RNA Purification Mini kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). RT for RT-qPCR was performed with a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The real-time PCR reaction system was prepared using SYBR® Premix Ex Taq™ II (RR420A; Takara Biotechnology Co., Ltd.) and the qPCR assay was performed with the CFX96TM Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reaction system (20 μl) consisted of 10 μl SYBR Premix Ex Taq, 0.5 μl forward primer, 0.5 μl reverse primer, 2 μl cDNA and 7 μl double-distilled water (ddH$_2$O). The reaction protocol was as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 10 sec. All the reactions were performed in accordance with the manufacturer's instructions. The primer sequences (miR-144, CXCL11, GAPDH, U6) for qPCR are listed in Table I. The relative expression of CXCL11 (reference gene GAPDH) and miR-144 (reference gene U6) was analyzed using the $2^{-\Delta\Delta C_q}$ method (22).

Immunohistochemistry. Using an immunohistochemical assay kit (BosterBio, Wuhan, China) according to the manufacturer's instructions, the tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin, then cut into 5-μm sections. The slides were treated by a graded series of alcohol and finally rinsed with ddH$_2$O. After antigen retrieval, endogenous peroxidase activity was blocked with 3% hydrogen peroxide at room temperature for 10 min. Subsequently, the slides were blocked with 10% normal goat serum (C0265; Beyotime Institute of Biotechnology, Haimen, China) for 1 h, followed by incubation with 1:250 diluted CXCL11 primary antibody (ab9955, rabbit anti-human; Abcam, Cambridge, MA, USA) at 4°C overnight. After washing off the primary antibody, the second antibody (ab6721, goat anti-rabbit IgG; Abcam) was applied and incubated at room temperature for 1 h. After DAB staining (P0203; Beyotime Institute of Biotechnology) until the appearance of brown color, the nuclei were stained with hematoxylin (C0107; Beyotime Institute of Biotechnology) for 1 min. Subsequently, the slides were washed with ddH$_2$O, sealed with neutral gum and covered with coverslips. An optical microscope (TS100-F; Nikon Corporation, Tokyo, Japan) was used for cell observation: Brown staining was considered as positive; the slides were observed under a magnification of x10 and x40; five non-overlapping fields were randomly selected from each section and images were captured. The mean integrated optical density (IOD/area) of the images was measured by Image-Pro Plus v.6.0 software, which was used for semi-quantitative analysis.

Western blot analysis. Total protein was extracted with the E.Z.N.A.® Total DNA/RNA/Protein kit (Omega Bio-Tek Inc.). The BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) was applied for protein concentration detection. The protein samples were mixed with loading buffer (P0015; Beyotime Institute of Biotechnology) and boiled for 10 min;

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Primer	Sequences
CXCL11	F:5'-ATAGCCTTGGCTGTGATATTGTGTG-3'
	R: 5'-CCTATGCAAAGACAGCGTCCTC-3'
GAPDH	F: 5'-AGAAGGCTGGGGCTCATTTGC-3'
	R: 5'-ACAGTCTTCTGGGTGGCAGTG-3'
miR-144	F: 5'-ACACTCCAGCTGGGGGATATCATCAT ATACTGT-3'
	R: 5'-CTCAACTGGTGTCGTGGAGTCGGCA ATTCAGTTGAGCTTACAG-3'
U6	F: 5'-CTCGCTTCGGCAGCAC-3'
	R: 5'-AACGCTTCACGAATTTGCG-3'

F, forward primer; R, reverse primer; miR, microRNA.

then, 50 μg protein from each sample was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (EMD Millipore, Billerica, MA, USA). The blot was blocked with 5% non-fat milk at room temperature for 2 h and then incubated with CXCL11 primary antibody (ab9955, rabbit anti-human, Abcam, USA) for 20 h at 4˚C. After TBST washing, the membrane was incubated with secondary antibody (ab6721, goat anti-rabbit IgG; Abcam) at room temperature for 1 h. An ECL kit (P0018; Beyotime Institute of Biotechnology) was used for blot chemiluminescence and an exposing gel documentation system was applied for imaging the blots. Image Lab v.3.0 software was used to obtain and analyze the protein signal.

Enzyme-linked immunosorbent assay (ELISA). CXCLL11 in serum specimens was tested using a Human CXCL11 ELISA kit (ab187392; Abcam); 50 μl serum of each samples was added into appropriate wells for the detection. All the procedures were performed according to the manufacturer's instructions. The OD was measured at 450 nm with a microplate spectrophotometer (Multiskan™ FC Microplate Photometer; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell culture. 293T and HCT116 cells in this study were provided by the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and cultured with DMEM high-glucose medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% FBS (Shengong, Shanghai, China) at 37˚C in 5% CO_2.

CRC cell transfection. After growing to 50-70% confluence, HCT116 cells were transfected with agomiR-144 or agomiR-NC by using ExFect Transfection Reagent (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. AgomiR-144 and agomiR-NC were designed and chemically synthesized by Guangzhou RiboBio Co., Ltd., Guangzhou, China.

Dual-luciferase reporter assay. According to the bioinformatics analysis results, a fragment of the CXCL11 3'-untranslated region (UTR) containing the wild type (WT) or mutant (MT) seed regions of miR-144 was chemically synthesized *in vitro*. The products were inserted into Spe-1 and Hind III restriction sites of pMIR-REPORT luciferase reporter plasmids (Ambion; Thermo Fisher Scientific, Inc.). The constructs were co-transfected with agomiR-144 into 293T cells using ExFect® Transfection Reagent (Vazyme Biotech Co., Ltd.); the empty plasmid was set as control. After 48 h of transfection, the cells were harvested. The luciferase activity was measured using a dual-luciferase reporter assay kit (Promega Corporation, Madison, WI, USA) and GloMax 20/20 luminometer (Promega Corporation). All the procedures were performed according to the manufacturer's instructions.

Statistical analysis. IBM SPSS software (v.20.0; IBM Corp., Armonk, NY, USA) was used for statistical analysis. Data are expressed as mean ± standard deviation. Comparisons between two groups were performed using the Student's t-test, and multi-group measurement data were analyzed using one-way analysis of variance. The post hoc test was performed using Student-Newman-Keuls and Least Significant Difference-t test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression profile of CXCL11 in CRC tissues form microarray data analysis. To measure the expression of CXCL11 in CRC tissues, microarray datasets GDS4382, GDS4515, GDS3756 and GDS2947 were downloaded from GEO DataSets. The mean values of CXCL11 in CRC tissues were 3+fold higher compared with normal tissues (Fig. 1A); in microsatellite-unstable colorectal tumor tissues they were 2+fold higher compared with normal colonic mucosa tissues (Fig. 1B); in rectal tumor tissues they were 4+fold higher compared with normal rectal tissues (Fig. 1C); and in adenoma tissues they were 4+fold higher compared with normal mucosa tissues (Fig. 1D). All the above mentioned data supported the upregulation of CXCL11 in CRC tissues.

Expression of CXCL11 in the serum and tumor tissues of patients with CRC. RT-qPCR was used to determine the levels of CXCL11 mRNA in tumor tissue and serum samples; western blotting and immunohistochemistry were used to detect the expression and localization of the CXCL11 protein in tumor tissue samples; ELISA was conducted to measure the concentration of the CXCL11 protein in serum samples. The data revealed that the levels of CXCL11 mRNA were significantly upregulated in CRC tissues (2+fold higher, $P<0.05$; Fig. 2A) and serum samples (3+fold higher, $P<0.05$; Fig. 2D) collected from CRC patients. Upregulation of the CXCL11 protein was detected in CRC tissues (Fig. 2B and C) and the localization of CXCL11 was in the cytoplasm and on the cell membrane (Fig. 2C). Increased expression of the CXCL11 protein was also found in serum samples from patients with CRC (Fig. 2E). The expression trend of CXCL11 in clinical specimens was similar to our bioinformatics
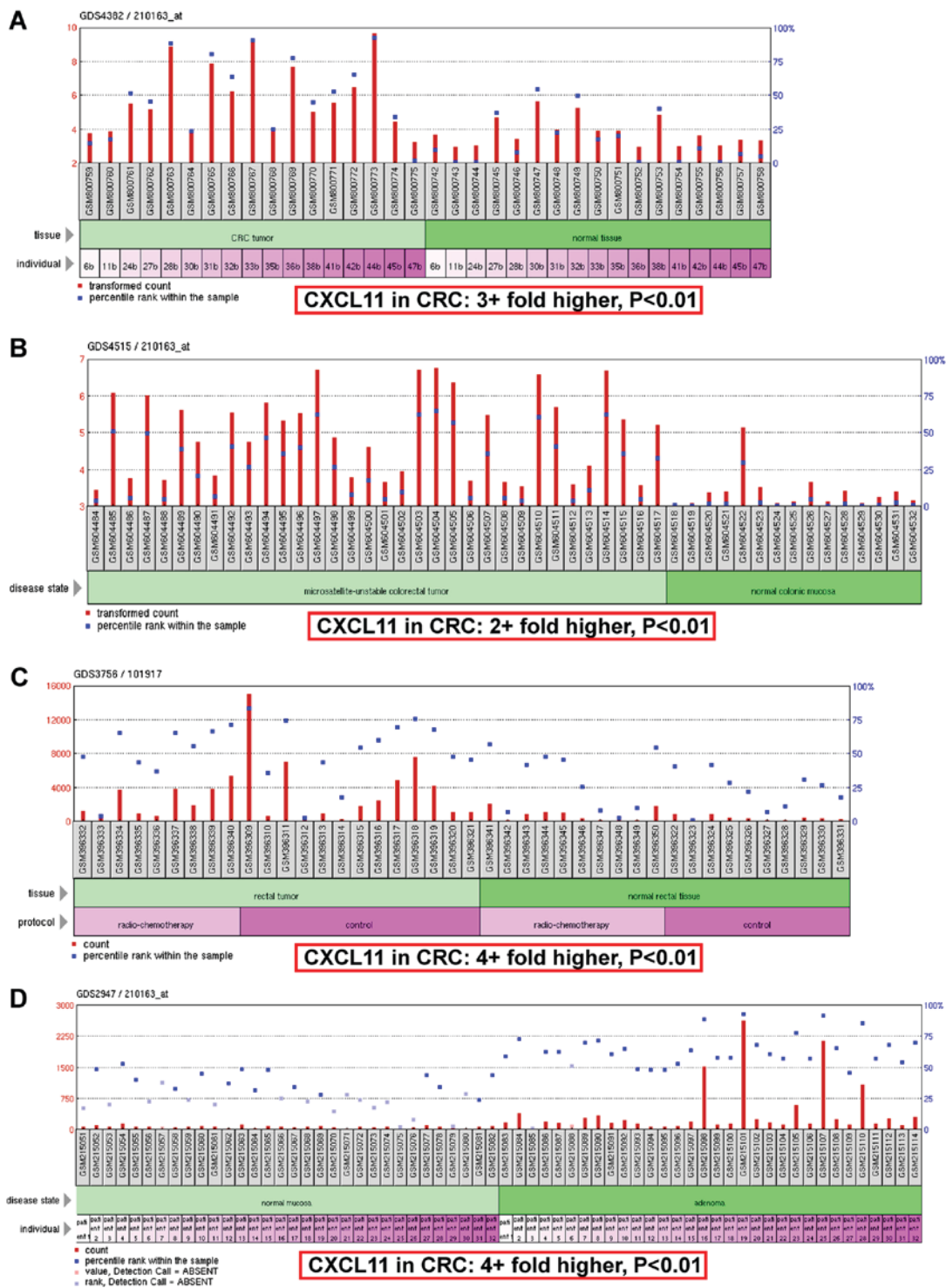
Figure 1. Expression profile of CXCL11 in CRC tissues was used to form microarray data analysis. Expression profile of CXCL11 in (A) GDS4382, microarray dataset of CRC; (B) GDS4515, microarray dataset of microsatellite-unstable CRC; (C) GDS3756, microarray dataset of rectal cancer; (D) GDS2947, microarray dataset of colorectal adenoma. The source of the data is the Gene Expression Omnibus database. CXCL11, C-X-C motif chemokine ligand 11; CRC, colorectal cancer.

analysis, indicating that increased CXCL11 expression may be implicated in CRC.

CXCL11 is a potential target of miR-144. To search for the potential regulator of CXCL11, miRWalk was used for the bioinformatics predictions. Of the 10 the established miRNA-target prediction programs, 6 predicted that CXCL11 was a potential target of miR-144 (Fig. 3A). The miR-144

target site in the CXCL11 mRNA 3'UTR was shown in some programs (Fig. 3B-D).

Expression of miR-144 in the serum and tumor tissues of patients with CRC. The levels of miR-144 in the serum and tumor tissues of patients with CRC were analyzed by RT-qPCR. Compared with adjacent tissues, miR-144 in CRC tissues was significantly reduced (P<0.05; Fig. 4A). The expression of
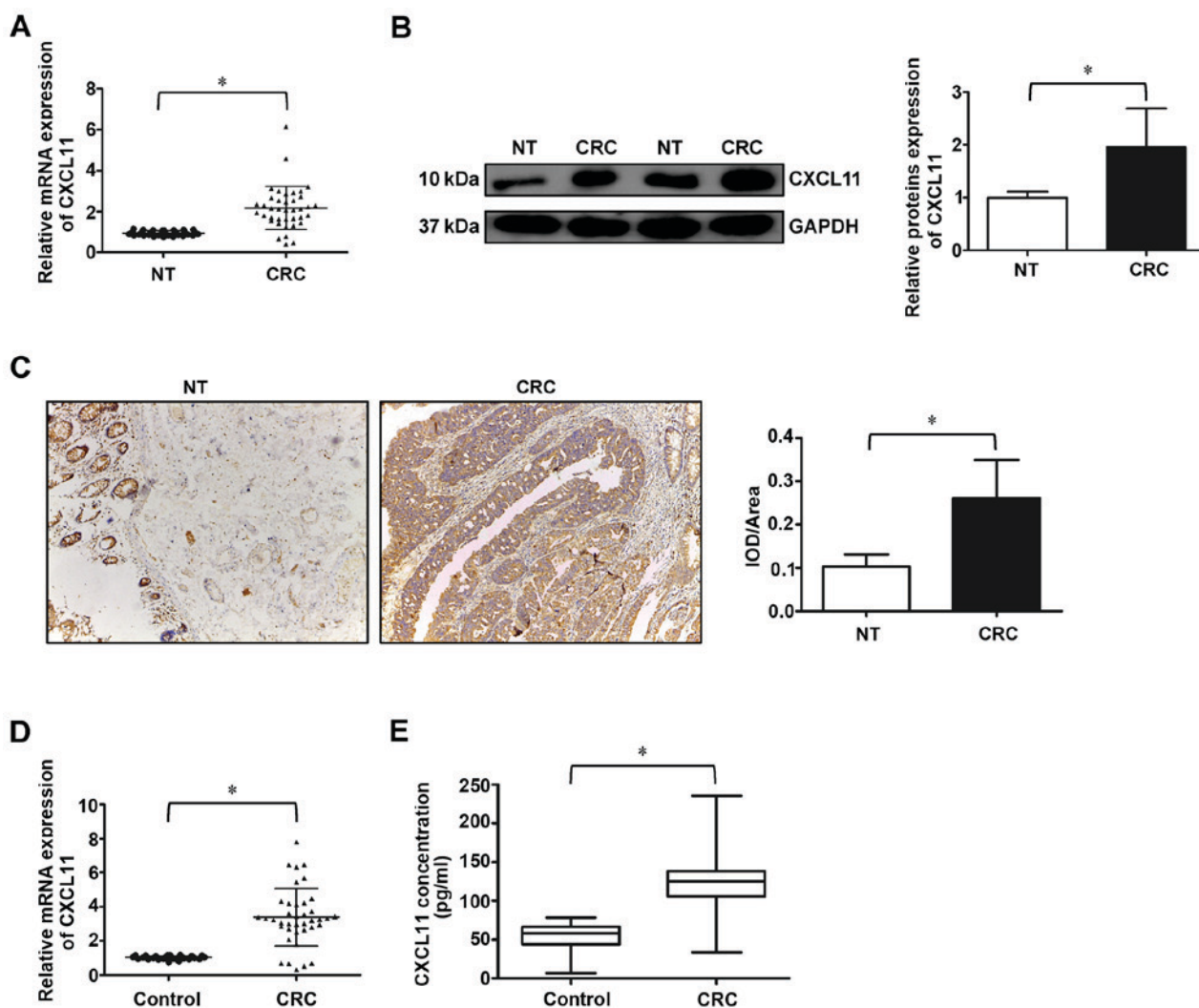
Figure 2. Expression of CXCL11 in tumor tissues and sera of patients with CRC. (A) Expression of CXCL11 mRNA in human CRC tissues. (B) Expression of CXCL11 protein in human CRC tissues. (C) Location and IOD/Area of CXCL11 in human CRC tissues (magnification, x100). (D) Expression of CXCL11 mRNA in serums of patients with CRC. (E) Levels of CXCL11 in serums of patients with CRC. *P<0.05 vs. corresponding control group (adjacent tissues from patients or serums from healthy subjects). CXCL11, C-X-C motif chemokine ligand 11; CRC, colorectal cancer; NT, adjacent non-tumor tissues.

miR-144 was also downregulated in the serum of CRC patients compared with the control subjects (P<0.05; Fig. 4B). Taking the expression of CXCL11 into account, these results indicated that miR-144 may be a negative regulator of CXCL11.

CXCL11 is a direct target of miR-144. To investigate the predicted interaction of miR-144 with CXCL11 mRNA, cell transfection and dual-luciferase reporter assay were used. HCT116 cells were selected for transfection with agomiR-144 or agomiR-NC *in vitro*. Compared with the agomiR-NC group, agomiR-144 significantly upregulated the expression of miR-144 (P<0.05; Fig. 5A), while it significantly downregulated the expression of CXCL11 (P<0.05; Fig. 5B and C) in HCT116 cells. WT and MT miR-144 binding sites were cloned into the pMIR-REPORT luciferase reporter plasmids (Fig. 5D) and co-transfected with agomiR-144 into 293T cells; the empty plasmid was used as control. Compared with the control group, transfection with agomiR-144 resulted in a significantly reduced fluorescence intensity in the WT group, but not in the MT group (Fig. 5E). These observations suggest that miR-144 may directly target the 3'-UTR of CXCL11 mRNA.

Discussion

The objective of the present study was to elucidate the role of miR-144 and CXCL11 in CRC progression. We reported the levels of CXCL11 in clinical specimens obtained from CRC patients. Then, using bioinformatics analysis, we found that miR-144 is a potential regulator of CXCL11. Next, we detected the expression of miR-144 in the serum and tumor tissues of CRC patients. Finally, luciferase assay verified that miR-144 directly targeted the 3'-UTR of CXCL11 mRNA.

First, differentially expressed genes in CRC tissues were identified from microarray data analysis. Microarray analysis has been applied to investigate the processes involved in CRC, as it is an effective tool for detecting general genetic alterations in the study of oncology (23,24). The most obvious finding to emerge from the analysis was the enhanced expression of CXCL11 in both CRC and colorectal adenomas, even up to 4-fold higher compared with normal tissues. Interferon-γ-inducible chemokines are crucial immunomodulators, not only in colon cancer, but also in other types of cancer, such as thyroid cancer (25).
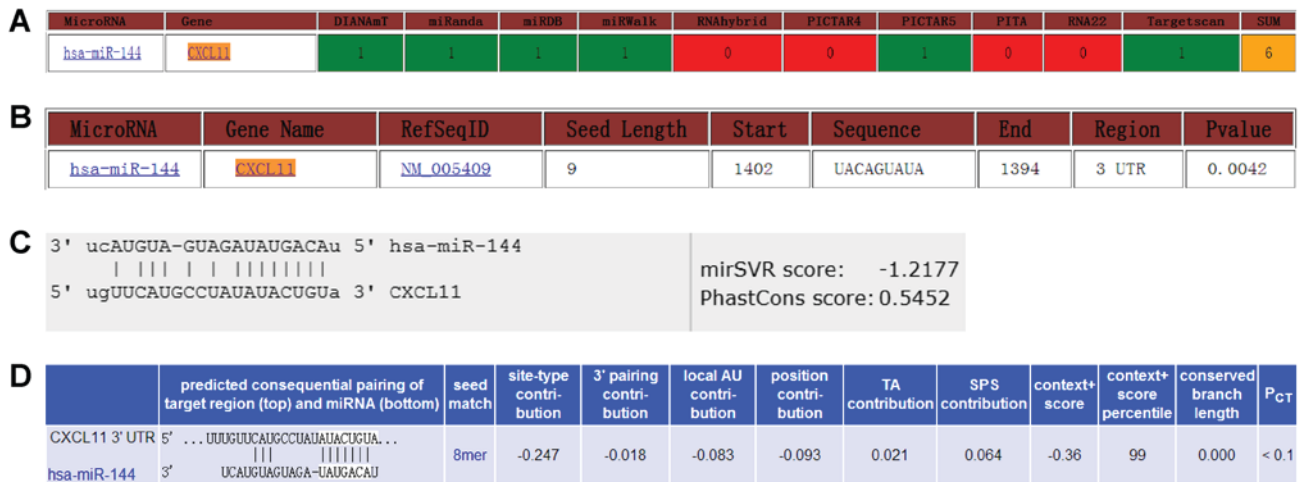
Figure 3. The potential regulator of CXCL11. (A) The prediction for miR-144 and CXCL11 in established miRNA-target programs from miRWalk. The predicted binding sites of miR-144 in CXCL11 mRNA 3'-UTR from (B) miRWalk, (C) miRanda and (D) TargetScan. CXCL11, C-X-C motif chemokine ligand 11.
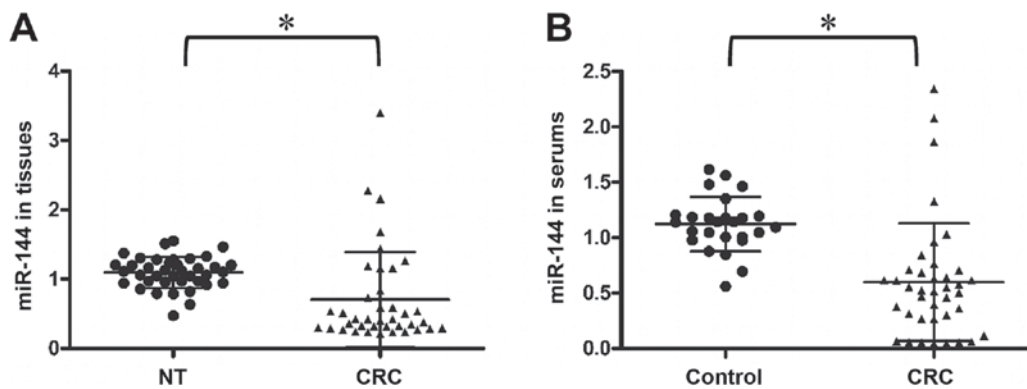

Figure 4. Expression of miR-144 in tumor tissue and sera of patients with CRC. (A) Expression of miR-144 in human CRC tissues. (B) Levels of miR-144 in serums of patients with CRC. *P<0.05 vs. corresponding control group (adjacent tissues from patients or serums from healthy subjects). CRC, colorectal cancer.

Other studies have also evaluated interferon-γ-inducible chemokines in colon cancer (26). Interferon-γ-inducible CXC-chemokines regulate angiogenesis and recruitment of immune cells in cancer progression (27), and they are closely associated with CRC. Not only CRC cell lines were demonstrated to release CXC-chemokines in response to cytokine stimulation, but also resected tumor explants were found to produce CXC-chemokines under stimulation, even in cases with initially low CXC-levels (26). Among these CXC-chemokines, CXCL11 is deemed to be an important factor during colonic tumorigenesis (5,28). Similar to the results of microarray data analysis, we found that the expression levels of CXCL11 were significantly upregulated in excised tumor specimens compared with normal tissues. However, there are no sufficiently clear mechanistic analyses on the regulation of CXCL11 in CRC. We herein provide evidence that CXCL11 is coordinately regulated by miR-144 in CRC.

It has been demonstrated that miRNAs are crucial for the occurrence and development of CRC (29,30). Through bioinformatics analysis, we identified CXCL11 as a potential target of miR-144. Previous miRNA profiling data revealed that miR-144 levels were significantly reduced in CRC (12,13). As a tumor suppressor, miR-144 may inhibit the malignant biological behavior of CRC by binding to its target mRNAs (31,32). Thus, we analyzed the levels of miR-144 in excised CRC tissues. The results revealed that the expression trends of miR-144 were contrary to those of CXCL11: Compared with normal tissues, the expression of miR-144 was significantly downregulated in tumor specimens. In CRC cells, upregulation of miR-144 also significantly downregulated the expression of CXCL11. Subsequently, the predicted association between miR-144 and CXCL11 was verified via the dual-luciferase reporter assay: Changes in relative fluorescence intensity induced by agomiR-144 indicated their interaction. This is a novel mechanism of miR-144 regulating the expression of CXCL11 by directly targeting the CXCL11 mRNA 3'-UTR seed sequence. Some other miRNAs such as miR-128 and miR-376, were also predicted to interact with CXCL11. We plan to study these miRNAs in future.

CXCL11 plays an important role in chronic inflammation, which is a common initiating step in tumorigenesis (23-35). CXCL11 may enhance the chemotaxis of T cells, particularly Th1 cells highly expressing CXCR3 (8); through the PI3K-AKT and RAS-MAPK pathways, CXCL11 promotes the proliferation and migration of tumor cells and exerts
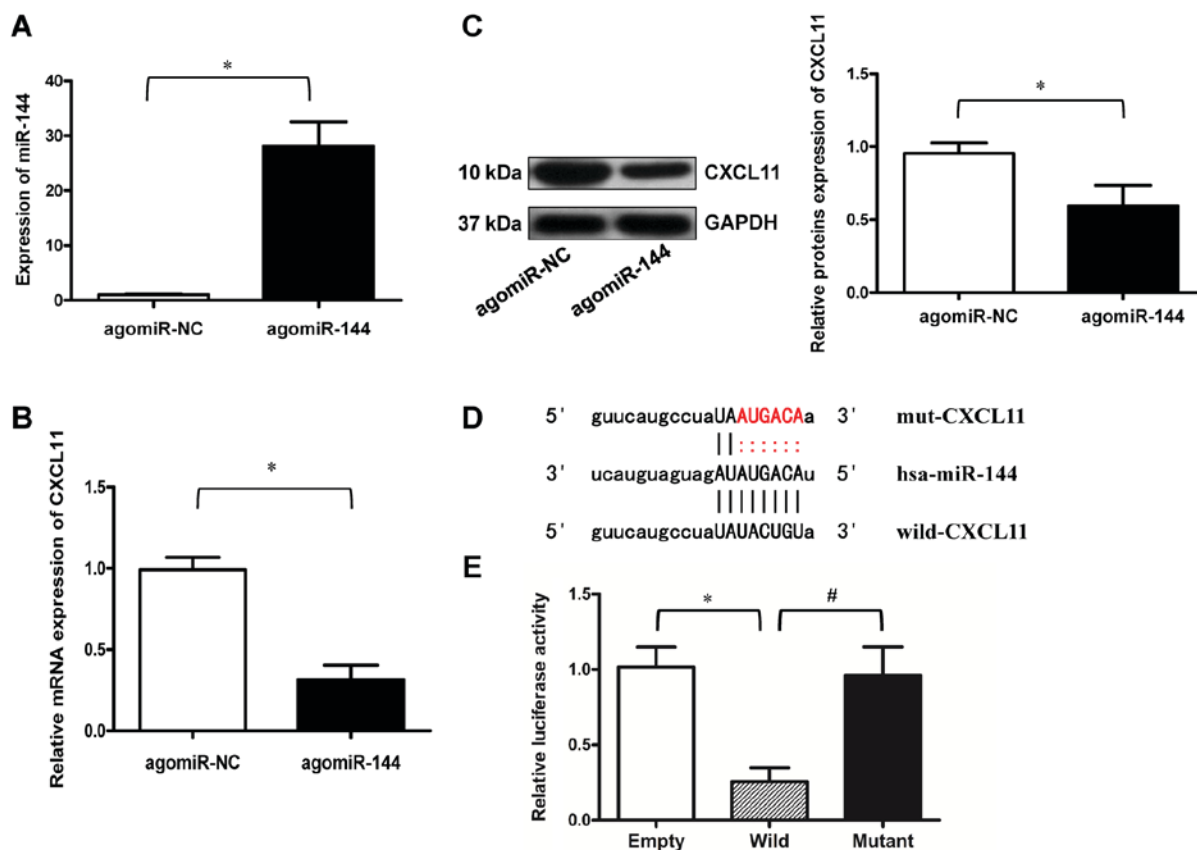
Figure 5. CXCL11 is a direct target of miR-144. (A) Expression of miR-144 in HCT116 cells. (B) Expression of CXCL11 mRNA in HCT116 cells. (C) Expression of CXCL11 protein in HCT116 cells. (D) Wild-type or mutant-type seed regions of miR-144 in CXCL11 3'-untranslated region (3'-UTR). (E) agomiR-144 was co-transfected with the pMIR-REPORT empty, wild-type or mutant-type CXCL11 3'-UTR vectors in 293T cells. The relative luciferase activity is reported. *P<0.05 vs. corresponding control group (agomiR-NC or empty vector), #P<0.05 vs. mutant-type vector. CXCL11, C-X-C motif chemokine ligand 11.

anti-apoptosis effects (36). CXCL11 also promotes tumor formation and development via binding with CXCR7 (37), and it may even be used as an independent prognostic factor for myeloma, along with CXCL9 and CXCL10 (38). These previous studies suggested that CXCL11 may be a key cytokine interlinking inflammation and tumor development. Moreover, as a regulator of CXCL11, miR-144 may affect not only colorectal tumorigenesis, but also chronic inflammation prior to CRC development.

Another novel finding of our study is that decreased levels of miR-144 are associated with increased levels of CXCL11 in the serum of patients with CRC, suggesting their potential value as biomarkers for CRC prediction. However, this study was conducted in a small sample size of cases and controls, and the findings require further evaluation in the future.

In conclusion, our study demonstrated that enhanced expression levels of CXCL11 in patients with CRC were associated with the decreased expression of miR-144. Through binding to the CXCL11 mRNA 3'-UTR, miR-144 may regulate the chronic inflammatory and tumorigenic processes in CRC.

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Availability of data and materials

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

Authors' contributions

BH and DF analyzed and interpreted the patient data, conceived and designed the study, and collected and analyzed experiment data. XY, YQL and MY read and analyzed the documents, and collected and analyzed data. FLi performed the experiments, critically revised the manuscript and gave valuable advice for the study. LMZ and FLu conceived and designed the study, read and analyzed the documents, drafted and revised the manuscript and gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed involving human participants were in accordance with the ethical standards of the

Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Tissue and blood samples were collected from consenting individuals according to the protocols approved by the Ethics Review Board at Sichuan University.

Consent for publication

The patient, parent, guardian or next of kin (in case of deceased patients) provided written informed consent for the publication of any associated data and accompanying images.

Conflict of interest

The authors declare that they have no competing interests.

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