Int J Clin Exp Pathol 2018;11(2):526-536
www.ijcep.com /ISSN:1936-2625/IJCEP0070504

Original Article

microRNA-9 functions as a tumor suppressor in colorectal cancer by targeting CXCR4

Wan-Cheng Xiong[1], Na Han[2], Guan-Fang Ping[3], Peng-Fei Zheng[1], Hai-Long Feng[1], Lei Qin[4], Peng He[1]

Departments of [1]General Surgery, [2]Central Laboratory, [3]Pharmacy, [4]Gastroenterology, First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan, China

Received December 6, 2017; Accepted December 27, 2017; Epub February 1, 2018; Published February 15, 2018

Abstract: MicroRNAs (miRs) dysregulation has been proven to play a crucial role in the initiation and progression of colorectal cancer (CRC). miR-9 functions as a tumor suppressor in many cancer types, including CRC. However, the precise role of miR-9 and the underlying molecular mechanisms that miR-9 involves in CRC progression remain largely unknown. In this study, it was reported that miR-9 had lower expression in CRC tissue samples than in those matched adjacent non-tumor tissues. Deregulated miR-9 expression was inverse correlated with the TNM stage, lymph node metastasis, and prognosis of CRC patients. Ectopic miR-9 expression suppressed CRC cell proliferation, migration, and invasion. Dual-Luciferase Reporter Assay confirmed that C-X-C Motif Chemokine Receptor 4 (CXCR4) was a direct miR-9 target, and the effects of miR-9 were mimicked through CXCR4 depletion *in vitro*. CXCR4 rescue experiments further verified that CXCR4 is a functional target of miR-9. Animal xenograft assays also provided evidence that miR-9 functions as a tumor suppressor via targeting CXCR4 *in vivo*. Mechanistically, miR-9 overexpression or CXCR4 knockdown influenced cell proliferation and epithelial-mesenchymal transition (EMT). Results suggest that miR-9 acts as a tumor suppressor in CRC progression by regulating CXCR4.

Keywords: miR-9, CXCR4, proliferation, migration, invasion

Introduction

Colorectal cancer (CRC) is a commonly diagnosed malignancy with approximately one million new diagnoses and over 693,900 annual deaths worldwide [1, 2]. Despite the efficient prognosis of CRC through surgical resection combined with chemotherapy or radiotherapy [3], distal metastases, and tumor recurrence after surgical resection may substantially yield a high mortality rate [4]. Therefore, identifying the molecules involved in CRC tumorigenesis may provide novel potential targets for clinical therapies.

MicroRNAs (miRs) are a class of small endogenous non-coding RNAs that regulate gene expression by base-pairing with the 3'-untranslated regions (UTRs) of target mRNAs and consequently inducing target mRNAs degradation or inhibiting protein expression [5, 6]. miR dysfunction is involved in tumor development and progression [7], and numerous miRs serve as oncogenic or tumor suppressors in different tumor phenotypes [8]. miR-9 is frequently downregulated in multiple cancer types and acts as a tumor suppressor in CRC [9-16]. However, the precise mechanism of miR-9 in the malignant phenotype of CRC remains unclear.

This study confirms that miR-9 is downregulated in CRC tissues and is correlated inversely with TNM stage. *In vitro* assays revealed that miR-9 restoration significantly inhibited CRC growth and metastasis. CXCR4 was also identified as a direct and functional target of miR-9, whose overexpression inhibited CRC tumorigenesis and progression in vivo. The inhibitory effects of miR-9 were phenocopied by silencing CXCR4 *in vitro* and *in vivo*. These results suggest that miR-9 participates in CRC by targeting CXCR4 and might serve as a potential therapeutic target of CRC.

Materials and methods

Ethics statement

Experimental procedures were approved by the Institutional Review Board and Ethics Committee of the First Affiliated Hospital of

Xinxiang Medical University (Xinxiang, China). Animal experiments were approved by the Institutional Committee for Animal Research and performed in accordance with the national guidelines for the care and use of laboratory animals.

Patients and tissue samples

CRC tissues and matched adjacent non-tumor tissues were collected from 160 CRC patients diagnosed histopathologically and subjected to surgical resection at the First Affiliated Hospital of Xinxiang Medical University between September 2014 and September 2016. None of the patients received radiotherapy, chemotherapy, or any other anticancer treatment before surgery. Clinical staging was performed according to the American Joint Committee on Cancer Staging Manual. All of the tissue samples were collected at surgery, immediately frozen in liquid nitrogen, and stored at -80°C until RNA or protein extraction. Written informed consent and approval were obtained from each patient or family.

Cell culture

Five human CRC cell lines (LOVO, SW620, HT29, DLD-1, and HCT116) and a normal colonic cell line (NCM460) were obtained from the Shanghai Institute for Biological Sciences (Shanghai, China) and American Type Culture Collection (Manassas, VA, USA). LOVO-luc CRC cells stably expressing highly efficient luciferase were purchased from PerkinElmer (Santa Clara, CA, USA) and characterized by the provider through gene profiling analysis. All of the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The cells were routinely grown to 80% confluence at 37°C in a humidified atmosphere containing 5% CO_2, and the cells from passages 2 to 4 were used in the experiments and expended no later than 6 months after receipt.

Cell transfection and infection

miR-9 mimic, siCXCR4 and their negative control RNAs were synthesized by Qiagen (Hilden, Germany). Transfection was performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. LOVO and HCT116 cells were transfected with 100 nM miR-9 mimics/miR-NC or siCXCR4 in six-well plates for the in vitro experiments. Lentivirus pGCsi and pGCsi-miR-9 were purchased from GenePharma and infected with LOVO-luc cells. The constructs were confirmed by DNA sequencing. After 12 days of screening with puromycin (5 μg/mL; Sigma), stable clones were generated and harvested for the CRC xenograft assays.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The total RNA of the fresh tissues and cell lines was extracted by utilizing TRIzol Reagent (Invitrogen). Reverse transcription was performed by using SuperScript™ II Reverse Tr anscriptase (Invitrogen), according to the manufacturer's protocol. cDNAs were detected with SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). mRNA expression was detected with SYBR Premix Ex Taq II (TaKaRa, Dalian, China) through qRT-PCR analyses. The total RNA was reverse-transcribed with a miScript Reverse Transcription Kit (Qiagen) and then amplified with SYBR Premix Ex Taq™ (TaKaRa) to quantify the miRs. $2^{-\Delta\Delta CT}$ method was applied to determine the relative gene expression. The expression levels of mRNAs and miRs were normalized to those of GAPDH and U6, respectively. The primers used in this experiment were as follows: 5'-GAGGCCCGTTTCTCTCTTTG-3' (forward) and 5'-AGCTTTATGACGG CTCTG TG-3' (reverse) for miR-9; 5'-GGTGGTCTATGT-TGGCGTCT-3' (forward) and 5'-TGGAGTGTG-ACAGCTTGGAG-3' (reverse) for CXCR4 and 5'-GCAC CGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGA-3' (reverse) for GAPDH.

Dual-luciferase reporter assay

Plasmid pGL3-CXCR4-3'-UTR-WT or pGL3-CXCR4-3'-UTR-MUT was co-transfected with 100 ng of miR-9 mimics and miR-NC in the cells by using Lipofectamine 2000 (Invitrogen Life Technologies). A luciferase reporter gene assay was conducted in the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI), according to the manufacturer's instructions. Renilla luciferase was co-transfected as a control for normalization.

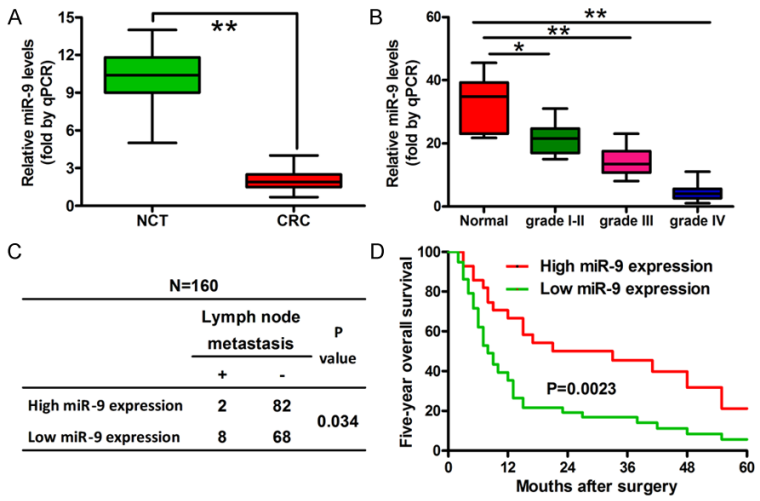

Figure 1. miR-9 levels in CRC tissues and the association between miR-9 expression and clinicopathological characteristics of CRC. (A) qPCR analysis of miR-9 expression in CRC and adjacent non-cancerous tissues (NCT). U6 was used as an endogenous control. (B) The miR-9 level in the tissue samples from patients of different TNM stages. (C) The association between miR-9 expression and lymph node metastasis. (D) Kaplan-Meier survival curves of 60 CRC patients divided by miR-9 levels. Data are shown as means ± SD of three separate experiments. **P < 0.01 compared with the NCT group (A, B).

Western blot analysis

Total protein was extracted from the cells or tissues by using a RIPA lysis buffer (Beyotime, Jiangsu, China) and quantified with a BCA protein assay kit (Bio-Rad, China). The proteins were separated through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Burlington, MA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated with primary antibodies targeting CXCR4, E-cadherin, Vimentin (1:2000; Abcam, Cambridge, UK), cyclin D1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-β-actin antibody (1:3000; Santa Cruz Biotechnology) in TBS overnight at 4°C. The blots were then washed and incubated for 1 hour with goat anti-mouse IgG conjugated to horseradish peroxidase (1:3000, Santa Cruz Biotechnology) in TBS for 1 hour at room temperature. Protein bands were detected through enhanced chemiluminescence (ECL) by utilizing a Pierce ECL Western blot substrate (Thermo Fisher Scientific) and exposed to an x-ray film by using an ECL detection system (Thermo Fisher Scientific). β-actin served as a control.

Cell viability detection

Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used to detect cell viability. LOVO or HCT116 cells were transfected with miR-9 mimics or siCXCR4, or co-transfected with miR-9 mimics and CXCR4, and then seeded into 96-well plates at a density of 5 × 10^3 cells/well. Cell viability was detected in 6 wells per group and in blank controls. Afterward, 10 μL of the CCK-8 solution was added at 0, 24, 48, 72, and 96 hours, and the resulting solution was incubated for 3 hours at 37°C. Optical density was measured at 450 nm to indicate the cell viability.

Colony formation assay

A total of 1 × 10^3 LOVO or HCT116 cells transfected with miR-9 mimics or siCXCR4-expressing plasmid, or co-transfected with miR-9 mimics and CXCR4, were plated in 3 cm plates precoated with 1% agar (Sigma) and cultured for 14 days. After the cells were fixed in 70% ethanol, the colonies were stained with the Diff-Quick Stain Kit (Yeasen Biotechnology, Shanghai, China) and counted under a microscope (Olympus, Tokyo, Japan). Five random fields were selected for each well to determine the total number of colonies.

5-ethyl-2'-deoxyuridine (EdU) incorporation assay

Cell proliferation was determined through EdU incorporation staining (RiboBio, Guangzhou, China), as described previously [17]. The cells were cultured in a medium with 20 μM EdU in 24-well plates for 2 hours and then fixed in 4% paraformaldehyde for 10 minutes at room temperature, according to the manufacturer's instructions. After the cells were incubated with Apollo solution and stained with Hoechst 33342, six random fields were selected to observe each well and were photographed under an inverted fluorescent microscope (Carl-Zeiss, Berlin, Germany).

Migration and invasion assay

Cell motility was assessed by using transwell chambers. For the migration assays, 1×10^4 cells plated in a serum-free medium were added to the upper chamber of 8 μm pore size Transwells (BD Biosciences, Franklin Lakes, NJ, USA). DMEM containing 100 ng/mL CXCL12 was then added to the lower chamber serving as a chemoattractant. For the invasion assays, 5×10^4 cells were added to the upper chamber of 8 μm pore size Tranwells precoated with 30 μl of Matrigel (BD Biosciences) at 200 μg/mL. After the preparations were incubated at 37°C for 24 hours, the cells that did not migrate or invade through the pores were carefully removed. The filters were then fixed in methanol, stained with crystal violet and counted. Three invasion chambers were utilized per condition, and five random fields were counted per chamber under an inverted microscope (Carl-Zeiss).

Animal xenograft assay

An animal xenograft assay was performed, as described previously. The hind flanks of 5 to 7 week old female severe combined immunodeficient (SCID) mice (Vital River Laboratory Animal Center, Beijing, China) were injected subcutaneously with 1×10^6 LOVO-luc cells infected with a control lentivirus or a recombinant lentivirus expressing an miR-9 precursor or shCXCR4 (n = 6 mice/group). Tumor growth was evaluated through *in vivo* luciferase imaging of the xenografts 7, 14, and 42 days after the treatment. *In vivo* luciferase imaging was performed by intraperitoneally injecting the mice with D-luciferin (Promega, Madison, WI, USA) at a dose of 150 mg/kg per mouse. The mice were anesthetized, and images were taken with a Xenogen IVIS imaging system. The signals in the defined regions of interest were quantified as a photon flux (photons/s/cm^2) by using Living Image (Xenogen Corporation, Berkeley, CA, USA). Tumor volume was monitored and calculated as follows: tumor volume = width^2 × length/2. All of the mice were sacrificed by euthanasia at 8 weeks post-inoculation, and the tumors were removed and weighed.

The infected LOVO-luc cells were used for *in vivo* metastasis assays (n = 6 mice/group). Five to seven week old female BALB/c mice were injected with 5×10^6 cells via the tail vein. The general health status of the mice and the signs of morbidity related to primary tumor or metastasis were monitored, and the mice were sacrificed by euthanasia at 8 weeks post-inoculation. The anatomized mice were examined to determine metastasis in the lungs. The lungs with visible tumor colonies were fixed and embedded in paraffin, and three non-sequential sections per mouse were obtained.

Statistical analysis

Data were statistically analyzed in SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Data from three independent experiments were presented as mean ± standard deviation (SD). One-way ANOVA among groups and two-tailed Student's t-test were performed to compare between groups. A Kaplan-Meier survival curve was plotted, and survival rates were compared. $P < 0.05$ was considered statistically significant.

Results

miR-9 is downregulated in CRC tissues and correlates with clinicopathological parameters and predicts prognosis

The miR-9 expression level was determined through qRT-PCR analyses and was normalized to that of an endogenous control (U6 RNA). **Figure 1A** shows that the miR-9 expression level is significantly lower in the CRC tissues than in the corresponding non-tumor tissues. We then examined the miR-9 expression level in the tissue samples from patients of different TNM stages. Our findings reveal that the miR-9 expression is decreased in advanced CRC (**Figure 1B**). Statistical analysis of 160 CRC cases shows that low miR-9 levels are significantly associated with lymph node metastasis, and this finding establishes the association between the miR-9 level and tumor metastasis of CRC patients (**Figure 1C**). A Kaplan-Meier survival curve was plotted to evaluate the prognostic significance of miR-9. Our results reveal that the decrease in miR-9 expression is significantly correlated with shorter median survival time (**Figure 1D**). Therefore, miR-9 could be considered an independent prognostic marker for the overall survival of patients with CRC.

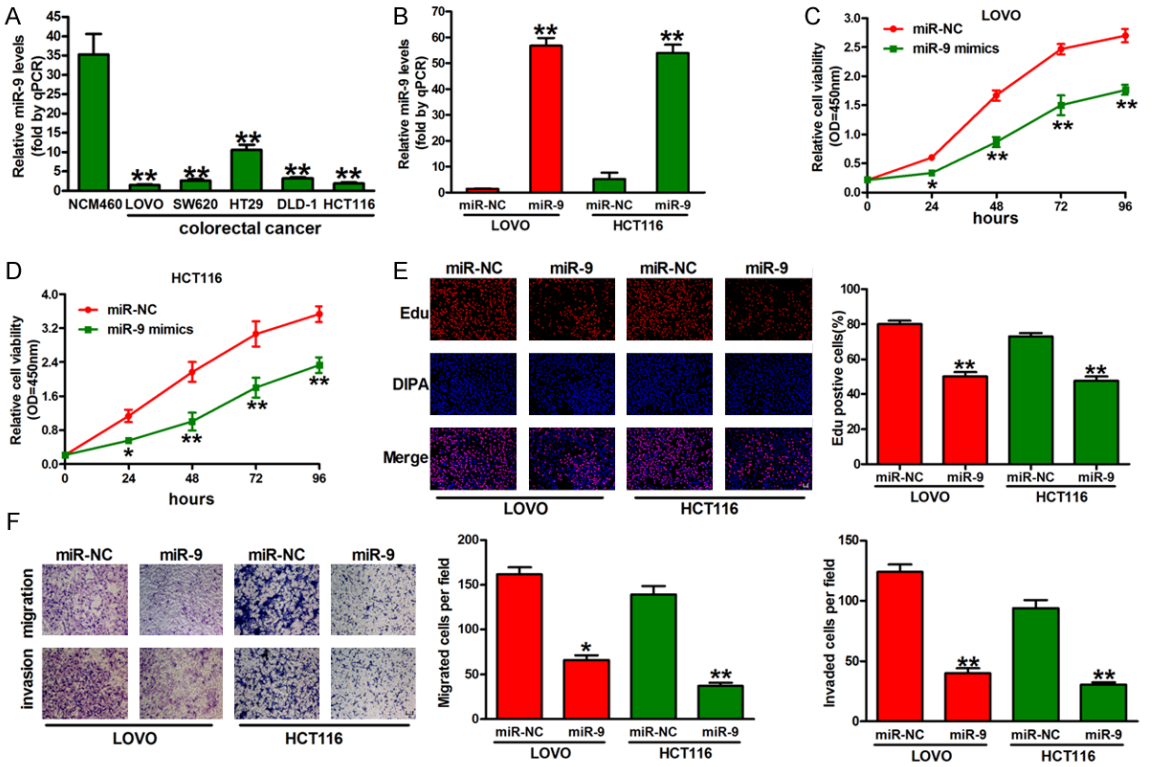

Figure 2. MiR-9 inhibits proliferation, migration, and invasion of CRC cells. (A) The miR-9 levels in five CRC cell lines and NCM460 cells. (B) The miR-9 level in LOVO and HCT116 cells transfected with miR-NC or miR-9 mimics. CCK-8 assay was performed to determine LOVO (C) and HCT116 (D) cell viability. (E) Representative photographs and the percentage of the EdU-positive cells. (F) Cell migration and invasion were determined by using Transwell chambers, and the percentage of migrated and invaded cells was calculated. Data are shown as means ± SD of three separate experiments. *P < 0.05 and **P < 0.01 compared with the miR-NC group.

miR-9 inhibits the proliferation, migration, and invasion of CRC cells

The miR-9 level in CRC cell lines were also measured, and our results reveal that the miR-9 expression is suppressed in the CRC cells compared with that in the normal colon cell NCM460 (**Figure 2A**). miR-9 mimics were used to change the miR-9 expression in CRC cells and to investigate the role of miR-9 in CRC tumor growth. In **Figure 2B**, miR-9 expression is significantly increased by miR-9 mimics in the LOVO and HCT116 cells. **Figure 2C** and **2D** illustrates that LOVO and HCT116 cell proliferation is significantly suppressed by miR-9 overexpression. Similarly, the percentage of EdU incorporation is decreased sharply in the miR-9-overexpressed (**Figure 2E**). Considering that miR-9 expression is associated with CRC metastasis, we examined the effect of miR-9 on cell invasion and tumor metastasis. The migration assay clearly demonstrates that miR-9 significantly suppresses CRC cell migration. The invasive capacity of CRC cells was determined by the Transwell assay and the results indicate that miR-9 markedly suppresses the invasion of the tested cell lines (**Figure 2F**). Therefore, miR-9 inhibits the proliferation and motility of CRC cells *in vitro*.

CXCR4 is a direct target of miR-9 in CRC cells

The candidate target genes of miR-9 were searched in a bioinformatics database (Target scan) to elucidate the molecular mechanisms of the anti-cancer effects of miR-9. On the basis of the results from the bioinformatics algorithm, we assumed that miR-9 might elicit an anti-tumor effect by targeting CXCR4. The potential binding sites of miR-9 in the 3'-UTR of CXCR4 are shown in **Figure 3A**. To investigate the correlation between the expression levels of miR-9 and CXCR4, we performed Dual-Reporter assays, and our findings reveal that

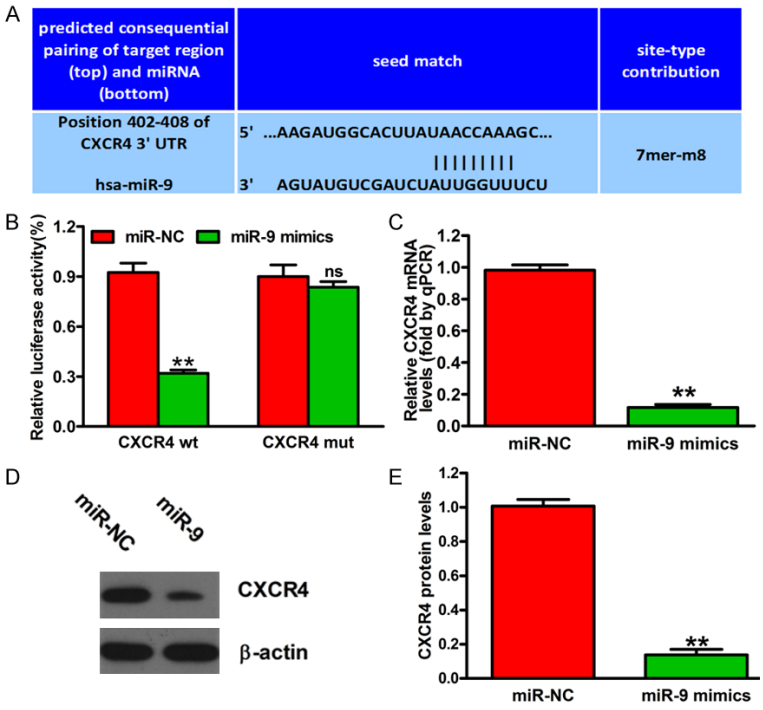

Figure 3. Identification of CXCR4 as a direct target of miR-9. (A) The predicted binding sites of miR-9 in the 3'-UTR of CXCR4 (positions 402-408) were predicted by utilizing TargetScan. (B) Dual-luciferase reporter assays were performed 24 hours after the co-transfection of cells with miR-NC or miR-9 mimics and a pGL3 construct that contains WT or MUT 3'-UTR of CXCR4. Data were normalized to those from cells co-transfected with miR-NC and pGL3 plasmid. The mRNA (C) and protein (D and E) levels of CXCR4 in the LOVO cells transfected with miR-NC or miR-9 mimics were measured by qPCR and Western blot assays. GAPDH and β-actin were utilized as internal controls. All data are shown as means ± SD of three separate experiments. **P < 0.01 compared with the miR-NC group.

the miR-9 introduction in LOVO cells inhibits the activity of a luciferase reporter fused to the wild-type (WT) 3'-UTR of CXCR4 but does not suppress the activity of a reporter fused to a mutant (MUT) version of the 3'-UTR (**Figure 3B**). The mRNA and protein levels of CXCR4 are also significantly inhibited by miR-9 introduction (**Figure 3C-E**). These results suggest that CXCR4 is a direct target of miR-9 in CRC cells.

CXCR4 mediates the effect of miR-9 in vitro

A high CXCR4 protein expression is associated with distant metastasis, clinical TNM stage, and poor survival [18]. These results indicate that miR-9 negatively modulates the CXCR4 expression at mRNA and protein levels by directly binding to its 3'-UTR. Thus, we hypothesized that CXCR4 is involved in miR-9-mediated CRC cell proliferation. siCXCR4 was used to knockdown the CXCR4 expression, evaluate its function in LOVO and HCT116 cells, and verify our hypothesis. In **Figure 4A** and **4B**, CXCR4 knockdown significantly attenuated the viability of LOVO and HCT116 cells. Consistent with this result, the findings obtained through colony formation assay confirm that the CXCR4 knockdown mimicked the miR-9-induced inhibitory effect on the cell formation rates in CRC cells (**Figure 4C**). The percentage of EdU incorporation was decreased sharply in the CXCR4-knocked down CRC cells compared with that of the siNC group (**Figure 4D**). Our results provide experimental evidence that CXCR4 mediated miR-9-induced antiproliferation effect. Cutler et al. reported that CXCR4 enables CRC metastasis [19]. We further determined whether CXCR4 mediates the anti-metastasis effect of miR-9. Our results indicate that CXCR4 knockdown significantly inhibits CRC cell migration and invasion, which partly phenocopies the inhibitory effect of miR-9 on the cell motility of CRC (**Figure 4E**).

Inhibition of malignant phenotypes of CRC cells by miR-9 is mediated by CXCR4

To confirm that CXCR4 is a functional target of miR-9, we performed CXCR4 rescue experiments. CXCR4 overexpression markedly counteracts the inhibition of cell viability of LOVO and HCT116 cells by miR-9 (**Figure 5A** and **5B**). Similarly, Edu incorporation staining showed that cell proliferation reduction caused by miR-9 restoration is inhibited by cotransfection with CXCR4-expressing plasmid (**Figure 5C** and **5D**). CXCR4 overexpression could partially counteract the decrease of cell migration and invasion of LOVO and HCT116 cells by miR-9 (**Figure 5E** and **5F**). These results indicate that CXCR4 is a key downstream mediator of miR-9 effects on CRC cells.

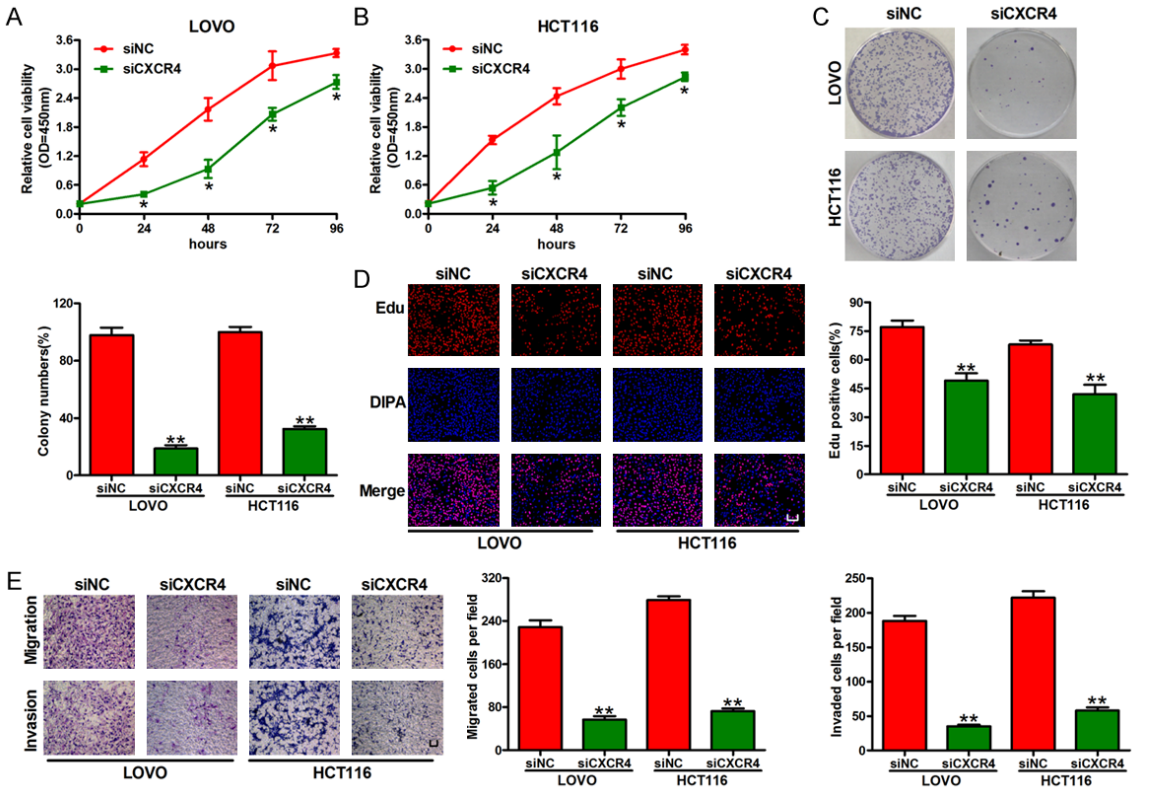

Figure 4. CXCR4 knockdown phenocopies the suppressive effects of miR-9 on the CRC cells. LOVO and HCT116 cells were transfected with siCXCR4 or its negative control plasmid. LOVO (A) and HCT116 (B) cell viabilities were determined through CCK-8 assay. (C) Representative photographs of the colony formation of LOVO and HCT116 cells and related quantitative analysis. (D) Representative photographs of the EdU-positive cells. Scale bar = 20 μm. (E) Representative photographs of migration and invasion assays and the percentage of migrated and invaded cells were calculated. Scale bar = 100 μm. *P < 0.05 siCXCR4 vs. siNC group.

miR-9 overexpression or CXCR4 knockdown suppresses tumor growth and CRC metastasis in vivo

The subcutaneous injection of LOVO-luc cells stably expressing a miR-9 precursor or shCXCR4 into the BALB/c mice produced tumors within 1 week. The tumor volumes were measured each week, and the mice were sacrificed 8 weeks after tumor cells were implanted. Using the Xenogen IVIS Imaging System, we observed that the growth of tumors derived from the LOVO-luc cells stably expressing miR-9 were lower than that of the tumors in the control group (**Figure 6A-C**). An *in vivo* metastasis model was then established by injecting the LOVO-luc cells with a stable ectopic miR-9 overexpression into the mice through the tail vein. The rate of lung metastasis was also lower in the xenograft tumors that overexpressed miR-9 than in the tumors derived from the control cells (**Figure 6D**). Similar to the effects of miR-9 overexpression, the CXCR4 knockdown inhibited the CRC tumor growth and lung metastasis (**Figure 6A-D**). Molecular analyses of the tumor tissues show that miR-9 overexpression or CXCR4 depletion reduces the levels of CXCR4, Cyclin D1 and Vimentin, whereas it upregulates the expressions of E-cadherin (**Figure 6E**). Hence, miR-9 might serve as a novel biomarker or therapeutic target for CRC.

Discussion

Despite significant progress in CRC diagnosis and therapy, the 5-year survival rate of patients with CRC remains essentially the same [4]. Therefore, novel prognostic factors and therapeutic strategies should be identified to improve the clinical outcome of patients with CRC. Although miR-9 has been identified as a tumor suppressor gene in CRC [15, 16], the

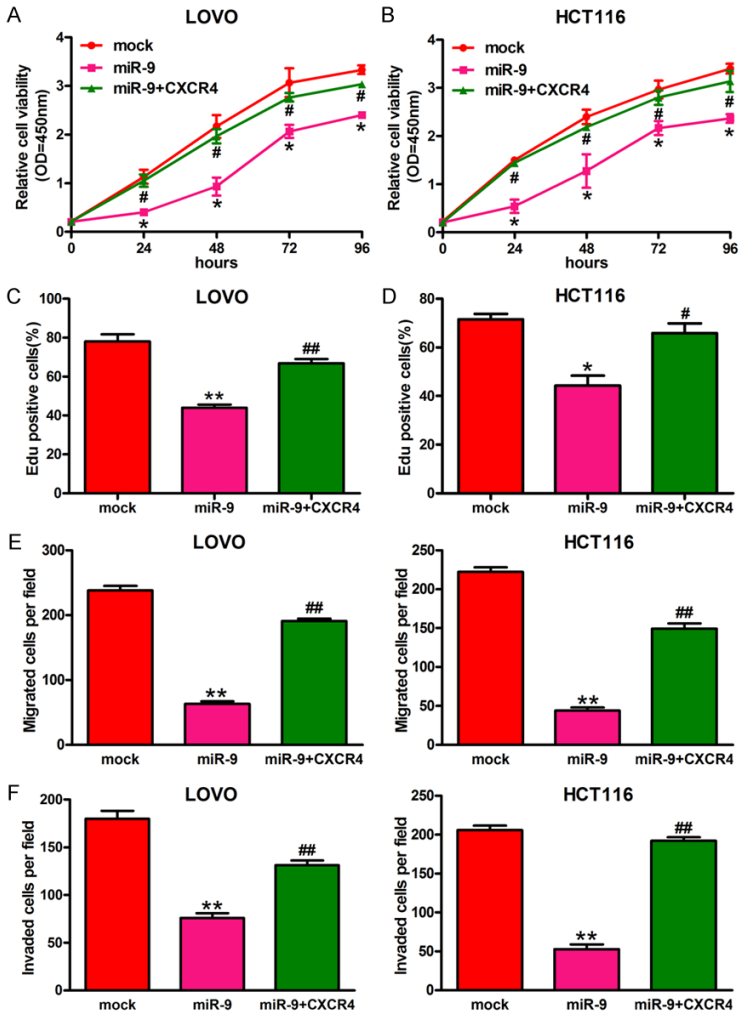

Figure 5. CXCR4 is a downstream functional mediator of miR-9 in CRC cells. Cells were transfected with miR-9 mimic or miR-9 mimic + CXCR4-expressing plasmid. LOVO (A) and HCT116 (B) cell viabilities were determined through CCK-8 assay. Representative quantitative analysis of EdU-positive cells of LOVO (C) and HCT116 (D) cells. The percentage of migrated (E) and invaded (F) cells was calculated. Data are shown as means ± SD of three separate experiments. *P < 0.05 **P < 0.01, miR-9 vs. mock group; #P < 0.05, ##P < 0.01, miR-9 + CXCR4 vs. miR-9 group.

underlying mechanism by which miR-9 inhibits CRC has yet to be fully investigated. Our study shows that miR-9 is downregulated in CRC tissues and cell lines and inversely associates with the TNM stage and lymph node metastasis. The induced miR-9 expression significantly decreases CRC cell proliferation, migration, and invasion. CXCR4 is identified as a direct target of miR-9. The CXCR4 knockdown mimicks the effects of miR-9 restoration on CRC cell motility *in vitro* and on tumor growth and metastasis in vivo. These data suggest that miR-9 might serve as a novel biomarker or therapeutic target for CRC.

The involvement of miR-9 in the development of human malignancies has been confirmed in preclinical and clinical studies [20]. We demonstrated that miR-9 expression was lower in CRC tissues than those in matched non-cancerous tissues, and this finding indicates its clinical significance. Consistent with other studies [21], our study reveals that miR-9 levels are inversely associated with TNM stage and lymph node metastasis, and this result suggests that miR-9 might function as a suppressor of CRC. Kaplan-Meier analysis showed that the 5-year overall survival rate of patients with high miR-9 levels is significantly higher than that of patients with low miR-9 levels and thus further confirms the potential clinical utility of miR-9. However, miR-9 is overexpressed in breast, bladder, hepatocellular, and cervical cancers, and its overexpression is correlated with cancer progression, metastasis, and poor prognosis [22-25]. These controversial results imply that miR-9 is expressed in a tissue-specific pattern and implicated in cell content-dependent functions.

Aberrant miR-9 expression regulates multiple CRC-related signaling pathways, such as cell proliferation, migration, and invasion [10]. Our results show that ectopic miR-9 expression effectively suppresses proliferation, migration, and invasion of CRC cells in LOVO and HCT116 cells. These findings are similar to those observed in gastric cancer [9, 10], melanoma [12], nasopharyngeal carcinoma [13], and oral squamous cell carcinoma [14]. In particular, miR-9 inhibits tumor growth and metastasis and acts as a putative tumor suppressor. Park YR et al. reported that miR-9 overexpression also inhibited the transwell migration and invasion of SW480 cells [16]. Conversely, Zhu L. et al.

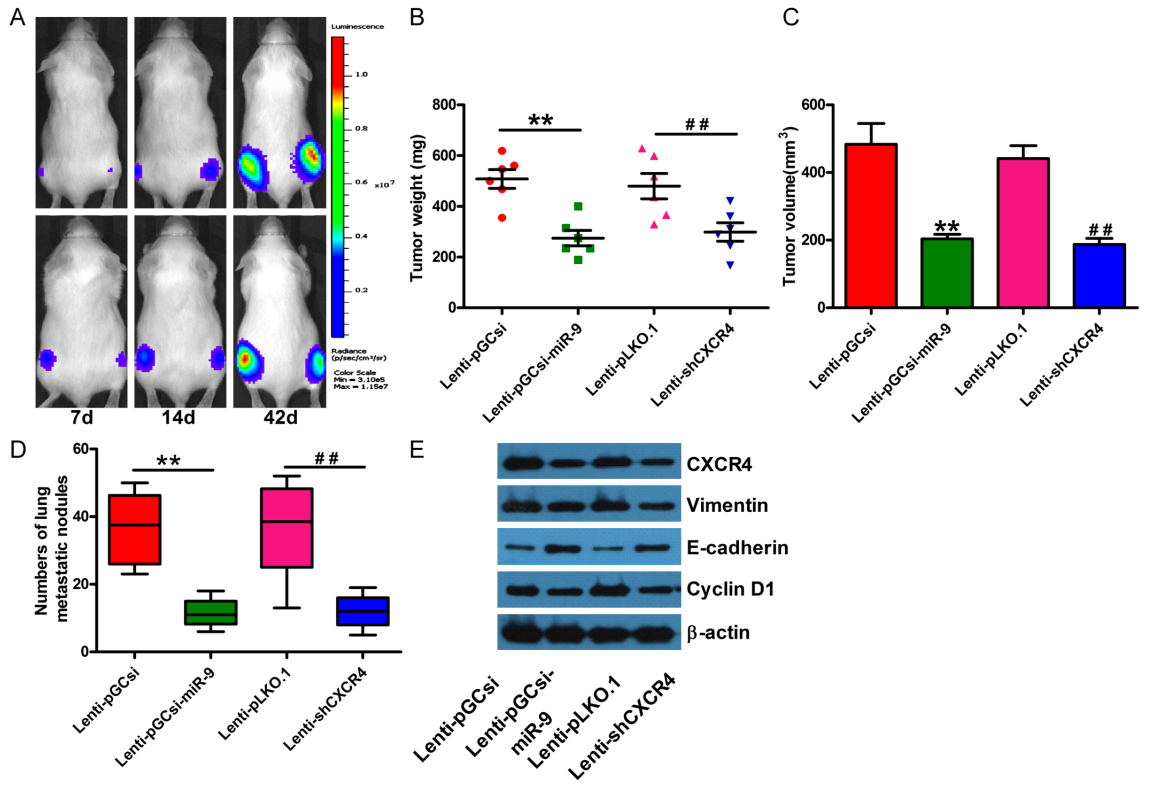

Figure 6. miR-9 overexpression suppresses tumor growth and metastasis of CRC cells in vivo. SCID mice were injected subcutaneously or through their tail veins with LOVO-luc cells infected with a control lentivirus (Lenti-pGCsi or Lenti-pLKO.1) or a recombinant lentivirus that expresses a miR-9 precursor (Lenti-pGCsi-miR-9) or shCXCR4 (Lenti-shCXCR4). (A) Tumor growth in the implanted mice was determined by *in vivo* luciferase imaging of the xenografts on day 7, 14 and 42. Upper panel: left hind, Lenti-pGCsi-miR-9; right hind, Lenti-pGCsi. Lower panel: left hind, Lenti-pLKO.1; right hind, Lenti-pLKO.1-shCXCR4. The tumor weights (B) and tumor volumes (C) of the subcutaneous CRC cell implantation models. (D) The numbers of metastatic foci in the lungs of mice from different groups at 8 weeks after tail vein injection. (E) Representative results of Western blot analyses of CXCR4, Cyclin D1, E-cadherin, and Vimentin in tumor tissues. β-actin was used as endogenous control. **P < 0.01 Lenti-pGCsi-miR-9 vs. Lenti-pGCsi group, ##P < 0.01 Lenti-shCXCR4 vs. Lenti-pLKO.1.

found that ectopic miR-9 expression enhanced the motility of RKO cells [26]. The discrepancies in these results could be attributed to different cell types, experimental conditions, and/or model systems utilized. miR-9 overexpression also suppresses tumor growth and metastasis *in vivo*. Therefore, miR-9 might be a potential diagnostic and prognostic marker of CRC patients.

A complex network of chemokines and their receptors influences the development of primary tumors and metastases [27]. CXCR4 activation induces tumor metastasis to distant organs via blood vessels in several tumor models [28]. CXCR4 is also involved in CRC metastases [29]. Therefore, it is a key receptor in the crosstalk between tumor cells and their microenvironment. Our study reveals the involvement of miR-9 in the progression of human CRC by regulating CXCR4 expression, which is similar to the findings in nasopharyngeal carcinoma [13] and oral squamous cell carcinoma cells [14]. Our study also indicates that CXCR4 is a novel target of miR-9 in CRC cells and CXCR4 knockdown inhibits cell proliferation, migration, and invasion. We further determined the downstream molecules of the miR-9/CXCR4 interaction. The downregulation of cyclin D1 could be a mechanism by which the miR-9 introduction or CXCR4 knockdown suppresses cell proliferation.

Epithelial-mesenchymal transition (EMT) is a hallmark of metastatic neoplasms, including CRC [30, 31]. In our study, the miR-9/CXCR4 interaction influenced CRC metastases by impeding the EMT of CRC cells significantly,

promoting the E-cadherin expression, and inhibiting vimentin expression. In particular, CXCR4 inhibitors have been regarded as effective anticancer targets that suppress the outgrowth of micrometastases in CRC [29]. Wnt/β-catenin signaling is also considered a potential regulator of EMT, stemness, and chemoresistance in colon cancer [30, 32]. Although our data strongly suggests that miR-9 overexpression or CXCR4 knockdown impedes EMT, whether miR-9/CXCR4-mediated signaling in colon cancer is involved in regulating the Wnt/β-catenin signaling pathway remains unknown. Thus, further investigation should be performed to identify additional signaling modulators and pivotal regulatory mechanisms of miR-9/CXCR4 in CRC progression.

In summary, this study demonstrates that miR-9 is consistently downregulated in CRC specimens and cell lines, and miR-9 expression is significantly and inversely associated with lymph-node metastasis and poor prognosis of CRC patients. Functional analyses identify miR-9 as a potential tumor suppressor in the development and progression of CRC by targeting the key metastasis promoter CXCR4. *In vivo* assays further confirm the inhibitory effect of miR-9 on CRC tumorigenesis via CXCR4. Our results provide evidence showing miR-9/CXCR4 as a potential therapeutic target in CRC treatment.

Acknowledgements

This work was supported by the Funding Scheme for Key Scientific Research Projects in Universities of Henan Province (No. 15A320060). The funders had no role in the study design, data collection and analysis, the decision to publish or preparation of the manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Peng He and Wan-Cheng Xiong, Department of General Surgery, The First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, Henan, China. Tel: 86 373-4404713; Fax: 86 373-4404713; E-mail: hepeng163001@163.com (PH); wanchengxiong2011@163.com (WCX)

References

[1] Chen W, Zheng R, Zeng H, Zhang S and He J. Annual report on status of cancer in China, 2011. Chin J Cancer Res 2015; 27: 2-12.

[2] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[3] Meyerhardt JA, Giovannucci EL, Holmes MD, Chan AT, Chan JA, Colditz GA and Fuchs CS. Physical activity and survival after colorectal cancer diagnosis. J Clin Oncol 2006; 24: 3527-3534.

[4] Fatemi SR, Pourhoseingholi MA, Asadi F, Vahedi M, Pasha S, Alizadeh L and Zali MR. Recurrence and five-year survival in colorectal cancer patients after surgery. Iran J Cancer Prev 2015; 8: e3439.

[5] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[6] Carthew RW and Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell 2009; 136: 642-655.

[7] Oliveto S, Mancino M, Manfrini N and Biffo S. Role of microRNAs in translation regulation and cancer. World J Biol Chem 2017; 8: 45-56.

[8] He L and Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004; 5: 522-531.

[9] Gao HY, Huo FC, Wang HY and Pei DS. MicroRNA-9 inhibits the gastric cancer cell proliferation by targeting TNFAIP8. Cell Prolif 2017; 50.

[10] Zheng L, Qi T, Yang D, Qi M, Li D, Xiang X, Huang K and Tong Q. microRNA-9 suppresses the proliferation, invasion and metastasis of gastric cancer cells through targeting cyclin D1 and Ets1. PLoS One 2013; 8: e55719.

[11] Emmrich S, Katsman-Kuipers JE, Henke K, Khatib ME, Jammal R, Engeland F, Dasci F, Zwaan CM, den Boer ML, Verboon L, Stary J, Baruchel A, de Haas V, Danen-van Oorschot AA, Fornerod M, Pieters R, Reinhardt D, Klusmann JH and van den Heuvel-Eibrink MM. miR-9 is a tumor suppressor in pediatric AML with t(8;21). Leukemia 2014; 28: 1022-1032.

[12] Liu S, Kumar SM, Lu H, Liu A, Yang R, Pushparajan A, Guo W and Xu X. MicroRNA-9 up-regulates E-cadherin through inhibition of NF-kappaB1-Snail1 pathway in melanoma. J Pathol 2012; 226: 61-72.

[13] Lu J, Luo H, Liu X, Peng Y, Zhang B, Wang L, Xu X, Peng X, Li G, Tian W, He ML, Kung H and Li XP. miR-9 targets CXCR4 and functions as a potential tumor suppressor in nasopharyngeal carcinoma. Carcinogenesis 2014; 35: 554-563.

[14] Yu T, Liu K, Wu Y, Fan J, Chen J, Li C, Yang Q and Wang Z. MicroRNA-9 inhibits the proliferation

of oral squamous cell carcinoma cells by suppressing expression of CXCR4 via the Wnt/beta-catenin signaling pathway. Oncogene 2014; 33: 5017-5027.

[15] Zhu M, Xu Y, Ge M, Gui Z and Yan F. Regulation of UHRF1 by microRNA-9 modulates colorectal cancer cell proliferation and apoptosis. Cancer Sci 2015; 106: 833-839.

[16] Park YR, Lee ST, Kim SL, Liu YC, Lee MR, Shin JH, Seo SY, Kim SH, Kim IH, Lee SO and Kim SW. MicroRNA-9 suppresses cell migration and invasion through downregulation of TM4SF1 in colorectal cancer. Int J Oncol 2016; 48: 2135-2143.

[17] Zhao H, Tang H, Huang Q, Qiu B, Liu X, Fan D, Gong L, Guo H, Chen C, Lei S, Yang L, Lu J and Bao G. MiR-101 targets USP22 to inhibit the tumorigenesis of papillary thyroid carcinoma. Am J Cancer Res 2016; 6: 2575-2586.

[18] Liu Y, Zhou Y, Feng X, Yang P, Yang J, An P, Wang H, Ye S, Yu C, He Y and Luo H. Low expression of microRNA-126 is associated with poor prognosis in colorectal cancer. Genes Chromosomes Cancer 2014; 53: 358-365.

[19] Cutler MJ, Lowthers EL, Richard CL, Hajducek DM, Spagnuolo PA and Blay J. Chemotherapeutic agents attenuate CXCL12-mediated migration of colon cancer cells by selecting for CXCR4-negative cells and increasing peptidase CD26. BMC Cancer 2015; 15: 882.

[20] Missiaglia E, Shepherd CJ, Aladowicz E, Olmos D, Selfe J, Pierron G, Delattre O, Walters Z and Shipley J. MicroRNA and gene co-expression networks characterize biological and clinical behavior of rhabdomyosarcomas. Cancer Lett 2017; 385: 251-260.

[21] Ahmed FE, Ahmed NC, Vos PW, Bonnerup C, Atkins JN, Casey M, Nuovo GJ, Naziri W, Wiley JE, Mota H and Allison RR. Diagnostic microRNA markers to screen for sporadic human colon cancer in stool: I. Proof of principle. Cancer Genomics Proteomics 2013; 10: 93-113.

[22] Gwak JM, Kim HJ, Kim EJ, Chung YR, Yun S, Seo AN, Lee HJ and Park SY. MicroRNA-9 is associated with epithelial-mesenchymal transition, breast cancer stem cell phenotype, and tumor progression in breast cancer. Breast Cancer Res Treat 2014; 147: 39-49.

[23] Wang H, Zhang W, Zuo Y, Ding M, Ke C, Yan R, Zhan H, Liu J and Wang J. miR-9 promotes cell proliferation and inhibits apoptosis by targeting LASS2 in bladder cancer. Tumour Biol 2015; 36: 9631-9640.

[24] Sun Z, Han Q, Zhou N, Wang S, Lu S, Bai C and Zhao RC. MicroRNA-9 enhances migration and invasion through KLF17 in hepatocellular carcinoma. Mol Oncol 2013; 7: 884-894.

[25] Hu X, Schwarz JK, Lewis JS Jr, Huettner PC, Rader JS, Deasy JO, Grigsby PW and Wang X. A microRNA expression signature for cervical cancer prognosis. Cancer Res 2010; 70: 1441-1448.

[26] Zhu L, Chen H, Zhou D, Li D, Bai R, Zheng S and Ge W. MicroRNA-9 up-regulation is involved in colorectal cancer metastasis via promoting cell motility. Med Oncol 2012; 29: 1037-1043.

[27] Itatani Y, Kawada K, Inamoto S, Yamamoto T, Ogawa R, Taketo MM and Sakai Y. The role of chemokines in promoting colorectal cancer invasion/metastasis. Int J Mol Sci 2016; 17.

[28] Zlotnik A, Burkhardt AM and Homey B. Homeostatic chemokine receptors and organ-specific metastasis. Nat Rev Immunol 2011; 11: 597-606.

[29] Verbeke H, Struyf S, Laureys G and Van Damme J. The expression and role of CXC chemokines in colorectal cancer. Cytokine Growth Factor Rev 2011; 22: 345-358.

[30] Brabletz T, Hlubek F, Spaderna S, Schmalhofer O, Hiendlmeyer E, Jung A and Kirchner T. Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. Cells Tissues Organs 2005; 179: 56-65.

[31] Bates RC and Mercurio AM. The epithelial-mesenchymal transition (EMT) and colorectal cancer progression. Cancer Biol Ther 2005; 4: 365-370.

[32] Huang D and Du X. Crosstalk between tumor cells and microenvironment via Wnt pathway in colorectal cancer dissemination. World J Gastroenterol 2008; 14: 1823-1827.