

Lentiviral vector-mediated IL-9 overexpression stimulates cell proliferation by targeting c-myc and cyclin D1 in colitis-associated cancer

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Abstract. Colorectal cancer caused by inflammatory bowel disease is referred as colitis-associated cancer (CAC). The mechanism underlying CAC is not fully understood. In the present study, the role of interleukin-9 (IL-9) in CAC was examined. The current study included 12 colorectal tissue specimens and matched adjacent tissues from CAC. The expression of IL-9 protein was examined using immunohistochemical staining. The expression of IL-9 in cancer tissues was markedly higher compared with that in adjacent tissues. Furthermore, IL-9 gene overexpression lentiviral vectors were constructed to overexpress IL-9 in RKO and Caco-2 cell lines. The role of IL-9 in cell proliferation was investigated using a Cell Counting Kit-8 assay, and MYC proto-oncogene bHLH transcription factor (*c-Myc*) and *cyclinD1* expression levels were detected by reverse transcription-quantitative polymerase chain reaction. Notably, IL-9 overexpression promoted the proliferation of colonic epithelial cells by upregulating of the expression of *c-Myc* and *cyclinD1*. In conclusion, the present results suggested that IL-9 may exhibit an essential role in the pathogenesis of CAC, and IL-9 promotes the proliferation of colonic epithelial RKO and Caco2 cells, partially via the upregulation of *c-Myc* and *cyclinD1* expression.

Introduction

Colorectal cancer is considered the third highest malignancy in the United States, causing >55,000 mortalities/year (1). Environmental and genetic factors influence the progression

of colorectal cancer, which is characterized by inflammation arising from various causes (2). The most common type of chronic intestinal inflammation associated with colorectal cancer is inflammatory bowel disease, which includes ulcerative colitis (UC) and Crohn's disease (CD). Colorectal cancer caused by inflammatory bowel disease is referred to as colitis-associated cancer (CAC).

CAC in patients with UC is caused by unifocal or multifocal dysplastic mucosa in chronic inflammatory regions (3). UC has been reported to increase the risk of CAC by approximately eight-fold in comparison to the general population (4). The degree and duration of the disease, and the number of flares have been recognized as risk factors, suggesting that uncontrolled inflammation may drive tumorigenesis (5). Chronic inflammation has been proposed to increase epithelial cell turnover in the colonic mucosa, resulting in accelerated aging of colonic mucosa, and enhanced vulnerability for acquiring genetic and epigenetic alterations (2). These results suggest that age-associated inflammation may be used for the identification of patients with UC with an increased risk of manifesting CAC later in life (6).

The molecular mechanisms underlying inflammation-mediated cancer development are not fully understood, and may differ between CAC and other forms of colorectal cancer. The involvement of cytokines and immune mediators is well understood in virtually all stages of colonic tumorigenesis, including tumor initiation, promotion, progression and metastasis (7). The pathogenesis of CAC is affected by several cytokines. Interleukin (IL)-9 forms part of the c-chain family of cytokines. Several types of cells receptive to IL-9 exist, including mast, T, antigen-presenting and epithelial cells, all of which are found in the human lung and gut (8). IL-9 also exhibits downstream regulatory activities on epithelial cells in the airway and intestinal mucosa barrier, where overexpression of IL-9 induces airway hyper-responsiveness and increases intestinal permeability (9). Previous research has recognized that IL-9 serves an important role in the pathogenesis of several chronic inflammatory and autoimmune diseases, including inflammatory bowel disease, multiple sclerosis, lupus erythematosus, food allergies and asthma (10). For instance, Gerlach *et al* confirmed that IL-9 expression is associated with UC, whereby IL-9 expression is markedly

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Abbreviations: IL-9, interleukin-9; CAC, colitis-associated cancer; UC, ulcerative colitis; MOI, multiple of infection

Key words: interleukin-9, colitis-associated cancer, *c-Myc*

increased in patients diagnosed with UC compared with healthy control participants and generally highest in patients with the more severe form of the disease (11). Based on these findings, we hypothesized that IL-9 inhibits the mucosal healing process, the subsequent exacerbation of inflammation and the formation of chronic lesions. In addition, IL-9 may mediate cell proliferation and serve catalytic role in CAC pathogenesis.

Inflammation is considered a cellular stressor that initiates DNA damage or genetic instability. Chronic inflammation may induce genetic abnormalities and epigenetic mechanisms, which can subsequently mediate malignant cell alteration. The nuclear transcription factor MYC proto-oncogene bHLH transcription factor (*c-Myc*) is part of the *Myc* gene family and has been reported to have multiple functions as an important oncogene (12). Given that it is an essential transcription regulator, the activation of *c-Myc* may accelerate cell cycle progression and induce cell apoptosis (13). *c-Myc* is upregulated in various types of malignant tumor, including colorectal cancer, and is considered compulsory for the uncontrolled proliferation of cancer cells (14). According to whole-exome sequencing analysis, the *c-Myc* genomic locus has been reported to be frequently expressed in CAC compared with sporadic colorectal cancer (15). Cyclins were named following their periodic cell cycle-dependent pattern of expression. Notably, *cyclin D1* is considered the most popular given its widespread role in human cancer with a larger depth of functional characterization. *Cyclin D1* is recognized as a proto-oncogene, whose overexpression is able to cause cell proliferation to become malignant and uncontrolled (16). Studies have reported the overexpression of *cyclin D1* gene in a variety of cancer types, including breast cancer, bladder cancer, parathyroid neoplasms, lymphoma, melanoma and lung cancer (17-22).

The present study aimed to examine the expression of IL-9 in CAC colorectal tissue specimens and the determine effect of IL-9 on cell proliferation. The results demonstrated that IL-9 was overexpressed in CAC tissues compared with adjacent tissues, and IL-9 overexpression promoted the growth of colonic epithelial cells by upregulating the expression of *c-Myc* and *cyclinD1*.

Materials and methods

Patients and tissue specimens. The present study was approved by the Research Ethics Committee of the First Hospital of Shanxi Medical University (Taiyuan, China). All patients agreed to participate and signed informed consent forms. The study used 12 (8 male and 4 female) colorectal tissue specimens from patients with CAC and tumor-adjacent tissues as a control. The median age of patients was 72.5 years (72.5±17.3 years) at the time of surgery. The specimens were collected from Department of Pathology at the First Hospital of Shanxi Medical University between June 2010 and December 2017. The diagnosis of CAC was founded on medical history, endoscopic findings, histological examination, laboratory tests and clinical disease presentation.

Immunohistochemistry. A total of 12 paired formalin-fixed, paraffin-embedded samples were subjected to immunohistochemistry staining for IL-9. Briefly, the cancer tissues were fixed

in 4% paraformaldehyde at 4°C for 48 h. Following dehydration, the tissues were sliced at 4 µm thickness and blocked in 10% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 30 min, followed by incubating with rabbit anti-human IL-9 monoclonal antibody (cat. no. A1894; 1:50-1:200; ABclonal Biotech Co., Ltd., Woburn, MA, USA) at 4°C overnight. The slices were incubated with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. SPN-9001; 1:100; OriGene Technologies, Inc., Rockville, MD, USA) at room temperature for 2 h. The SCANSOP Digital Pathology Scanning system (Aperio Technologies, Inc., Vista, CA, USA) was used for scanning of pathological slices. Under a light microscope, five representative high magnification fields of vision were selected for each slice, and the number of IL-9-positive immunization cells was counted. The mean value represents the IL-9 positive degree of the case.

Construction of lentivirus overexpressing IL-9. The gene sequence of IL-9 was based on National Centre for Biotechnology Information (NM_000590.1, https://www.ncbi.nlm.nih.gov/nucore/NM_000590.1), and the Basic Local Alignment Search Tool (BLAST) sequence was produced using DNAMAN software (version 6.0; Lynnon LLC, San Ramon, CA, USA). Overexpression lentiviral vector pGV367 and *AgeI/NheI* Restriction endonuclease were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). Human IL-9 was cloned from the human genome with the following primers: IL-9 forward, 5'-GAGGATCCC CGGGTACCGGTCGCCACCATGCTTCTGGCCATGGTC CTTAC-3' and reverse, 5'-CACACATTCCACAGGCTAGTC ATATCTTGCCCTCATCCCTCTCATC-3'. The lentivirus vector plasmid pGV367 and the IL-9 gene sequence were digested by *AgeI* and *NheI* restriction enzymes. Recombinant lentiviral vector was detected by DNA sequencing. Following exact matching of the sequencing, pGV367-IL-9 was cotransfected with two assistant plasmids pHelp 1.0 and pHelp 2.0 into 293 cells (purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) to establish the recombinant lentivirus expressing IL-9 (LV-IL-9). LV-negative control (NC) contains empty gene sequence was used as lentivirus control group. All the vectors were purchased from Shanghai GeneChem Co., Ltd.

Cell culture and LV-IL-9 transfection. Colorectal cancer cell RKO and Caco2 cells are adherent cells, and purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Caco2 cells were cultured in Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C with 5% CO₂. RKO cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (Life Technologies; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. RKO and Caco2 cells were infected with LV-IL-9 with a multiplicity of infection (MOI) of 10 and 20 respectively at 80% confluence. Subsequently, the expression of enhanced green fluorescent

protein (GFP) was observed by fluorescence microscope at a x200 magnification (IX71; Olympus Corporation, Tokyo, Japan).

Cell proliferation assay. Cell proliferation was investigated and determined using Cell Counting Kit 8 (CCK8; cat. no. AR1160-500; Wuhan Boster Biological Technology, Ltd., Wuhan, China). RKO and Caco2 cells were seeded into 96-well plates at a density of 2×10^3 cells/well in 100 μ l of culture medium, and infected with LV-IL-9 at a MOI of 20 or with LV-NC (2 μ l) (n=6) for 0, 12, 24, 48 and 72 h in a 5% CO₂ humidified incubator at 37°C. Subsequently, the cells were then incubated with 10 μ l of CCK8/well for 30 min at 37°C. The optical density of each well was assessed at 450 nm using a microplate reader.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from RKO and Caco2 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was reverse transcribed using Advantage[®] RT-for-PCR kit (cat. no. 639505; Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. Subsequently, the cDNA was subjected to RT-qPCR analysis. Amplification was performed as follows: Initial incubation at 95°C for 10 sec; followed by 40 cycles at 95°C for 5 sec and at 62°C for 45 sec; and extension at 72°C for 3 min. The 25 μ l PCR reaction system included 1 μ l cDNA template, 2 μ l primers, 2 μ l dNTP, 0.5 μ l RT/Platinum[™] Taqmix (Invitrogen; Thermo Fisher Scientific, Inc.) and distilled water. RT-qPCR was performed using the T100-Thermal Cycler Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Gene expression was normalized to the levels of the internal reference GAPDH using the $2^{-\Delta\Delta C_q}$ method (23). Each sample was assessed in triplicate and the mean expression level was determined. The following primers were used: *c-Myc* forward, 5'-TTCGGGTAGTGG AAAACCAG-3' and reverse, 5'-AGCAGATCGAATTTCTTCA-3'; *cyclinD1* forward, 5'-GAGGAAGAGGAGGAGGAGGA-3' and reverse, 5'-GAGATGGAAGGGGGAAGAG-3'; B cell lymphoma-2 (*Bcl-2*) forward, GGATGCCTTTGTGGA ACTGT-3' and reverse, 5'-AGCCTGCAGCTTTGTTTCAT-3'; Bcl-2 associated X protein (*Bcl-xL*) forward, 5'-TCTGGT CCCTTGCAGCTAGT-3' and reverse, 5'-ATTCTGAGGCCA AGGGAAGT-3'; *surviving* forward, 5'-ACCTGAAAGCTT CCTCGAGA-3' and reverse, 5'-AACCCTTCCCAGACTCCA CT-3'; induced myeloid leukemia cell differentiation protein Mcl-1 homolog (*Mcl-1*) forward, 5'-TGCTGGAGTAGGAGC TGGTT-3' and reverse, 5'-CCTCTTGCCACTTGCTTTTC-3'; *GAPDH* forward, 5'-CCACCTTCGATGCCGGGGCT-3' and reverse, 5'-GGGGCCGAGTTGGGATAGGG-3'.

Western blot analysis. The colorectal tissue specimens from patients with CAC and tumor-adjacent tissues were pulverized and lysed with RIPA buffer (Beyotime Institute of Biotechnology, Beijing, China) containing proteinase inhibitor cocktail (cat. no. P8340; Sigma-Aldrich; Merck KGaA). Protein concentration was measured using a bicinchoninic acid protein assay kit. A total of 60 μ g protein were subjected to 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Subsequently, the

membranes were blocked in 5% fat-free milk for 2 h at room temperature and incubated with the following primary antibodies overnight at 4°C: Rabbit polyclonal anti-IL9 (1:500; cat. no. ab111915; Abcam, Cambridge, UK) and rabbit monoclonal anti-GAPDH (1:500; cat. no. AF1186; Beyotime Institute of Biotechnology). Following incubation with the HRP-conjugated secondary antibodies from Santa Cruz Biotechnology, Inc. (1:2,000; cat. no. sc-2004, goat anti-rabbit IgG-HRP; cat. no. sc-2005) for 2 h at room temperature, the membranes were visualized using the ECLPlus kit (GE Healthcare, Chicago, IL, USA).

Enzyme-linked immunosorbent assay (ELISA). Concentrations of IL-9 were detected in the culture medium of RKO cells and Caco-2 cells using solid phase sandwich ELISA according to the manufacturer's protocols of ELISA kit (cat. no. BMS208196T, eBioscience; Thermo Fisher Scientific, Inc.). The hormone assay sensitivity was set at 0.1 pg/ml and the assay range was 3.1-200 pg/ml. For statistical analysis, the culture medium was independently collected three times. The ELISA kit for IL-9 was purchased from eBioscience (Thermo Fisher Scientific, Inc.).

Statistical analysis. All group data are presented as the mean \pm standard deviation. Comparisons among the three groups were performed using one-way analysis of variance, using the Fisher's least significant difference test for multiple comparisons. The Student's t-test was applied to analyze the differences between two groups. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

IL-9 expression is upregulated in CAC tissues. A total of 12 patients with CAC were enrolled for the present study, including eight males (66.7%) and four females (33.3%), with a mean age of 72.5 ± 17.3 years, and a history of ulcerative colitis (lasting 25.8 ± 3.8 years). The basic clinical information of the patients was obtained by consulting their disease history.

While IL-9 involvement in atopic diseases has been previously established (24), its expression in patients with CAC remains to be elucidated. To investigate the potential role of IL-9 in CAC, IL-9 expression levels were determined. Among the 12 cases of CAC, all had IL-9 expression in the intestinal mucosal epithelial cells and inflammatory cell cytoplasm (Fig. 1A). It was demonstrated that the number of IL-9-positive cells in the intestinal mucosa was significantly increased compared with that in paracancerous tissues (Fig. 1B; $P < 0.001$). Furthermore, the western blot analysis results demonstrated that IL-9 protein expression was significantly increased in CAC tissues (Fig. 1C; $P < 0.001$).

Identification of IL-9 gene overexpression using lentivirus vector. The BLAST results for the IL-9 cDNA sequence determined that the expected IL-9 gene sequence was completely consistent. It was confirmed that the IL-9 was correctly inserted into the vector and that the IL-9 overexpression lentivirus vector was successfully constructed. LV-IL-9-infected

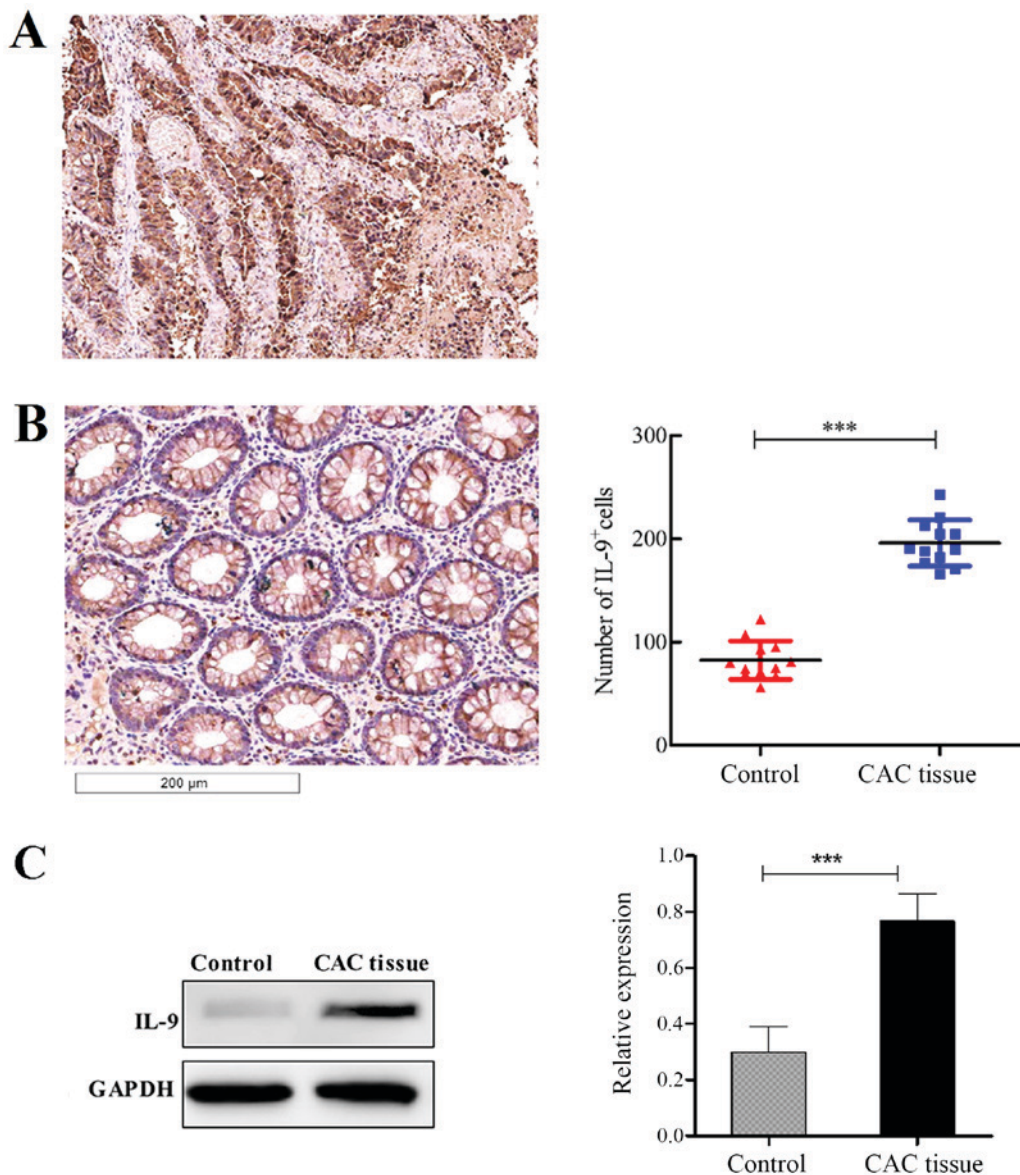


Figure 1. IL-9 is overexpressed in colonic biopsy specimens from patients with CAC. (A and B) Expression of IL-9 was examined by immunohistochemical staining of IL-9 in (A) CAC and (B) tumor-adjacent tissues. The number of IL-9-positive cells in the intestinal mucosa was significantly increased compared with that in paracancerous tissue. ***P<0.001. (C) Representative western blot and quantitative data demonstrating IL-9 expression in CAC and adjacent tissues. ***P<0.001. IL-9, interleukin-9; CAC, colitis-associated cancer.

293 cells were detected by fluorescence microscopy through examining the level of GFP-positive cells (Fig. 2A and B).

LV-IL-9 effectively infected colonic epithelial cells. GFP was observed in RKO and Caco2 cells under a fluorescence microscope indicating that the lentivirus has been successfully transfected (Fig. 3A). The RT-qPCR results demonstrated that IL-9 gene expression in the Caco-2 overexpression (OE) group was 12,508.689 times that of the NCgroup (Fig. 3B; P<0.001). Furthermore, IL-9 gene expression in the RKO OE group was 5,037.700 times that of the NC group (Fig. 3B; P<0.001). IL-9 protein expression was 141.61±12.28 pg/ml in the transfected Caco-2 cell medium as determined by ELISA, whereas IL-9 expression was 25.79±5.22 pg/ml in the untransfected group (Fig. 3C). IL-9 protein level was 106.65±17.45 pg/ml in the transfected RKO medium and 35.23±4.45 pg/ml in untransfected RKO cells (Fig. 3C). The IL-9 protein expression levels

were significant increased in the two OE groups compared with their untransfected counterparts (P<0.05).

IL-9 overexpression promotes cell proliferation. The CCK8 assay demonstrated that IL-9 overexpression induced a significant increase in the proliferation rates of Caco-2 and RKO cells compared with that of the corresponding NC groups (Fig. 4A and B).

IL-9 overexpression upregulates c-Myc and cyclinD1 mRNA expression. As presented in Fig. 5A and B, compared with the control and LV-NC groups, the relative levels of *c-Myc* and *cyclinD1* mRNA were demonstrated to be significantly increased in Caco-2 and RKO cells infected with LV-IL-9. However, no significant differences in the expression levels of Bcl-2, Bcl-xL protein, surviving or Mcl-1 were observed following IL-9 OE (Fig. 5C-F).

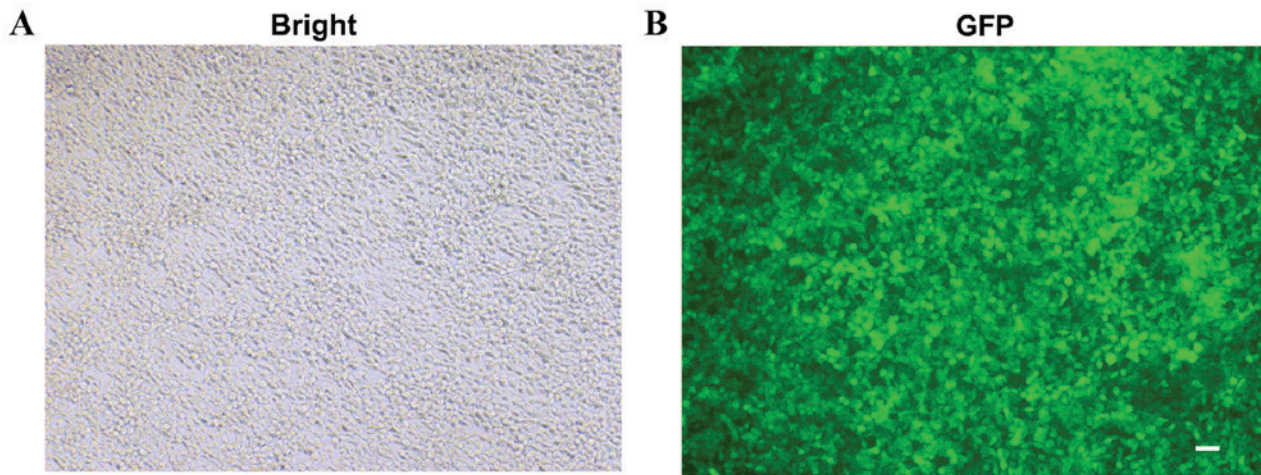


Figure 2. Lentiviral vector-interleukin-9 efficiently infects 293 cells. Infection efficiency as determined by (A) light and (B) fluorescence microscopy at 72 h postinfection in 293 cells. Scale bar, 50 μ m.

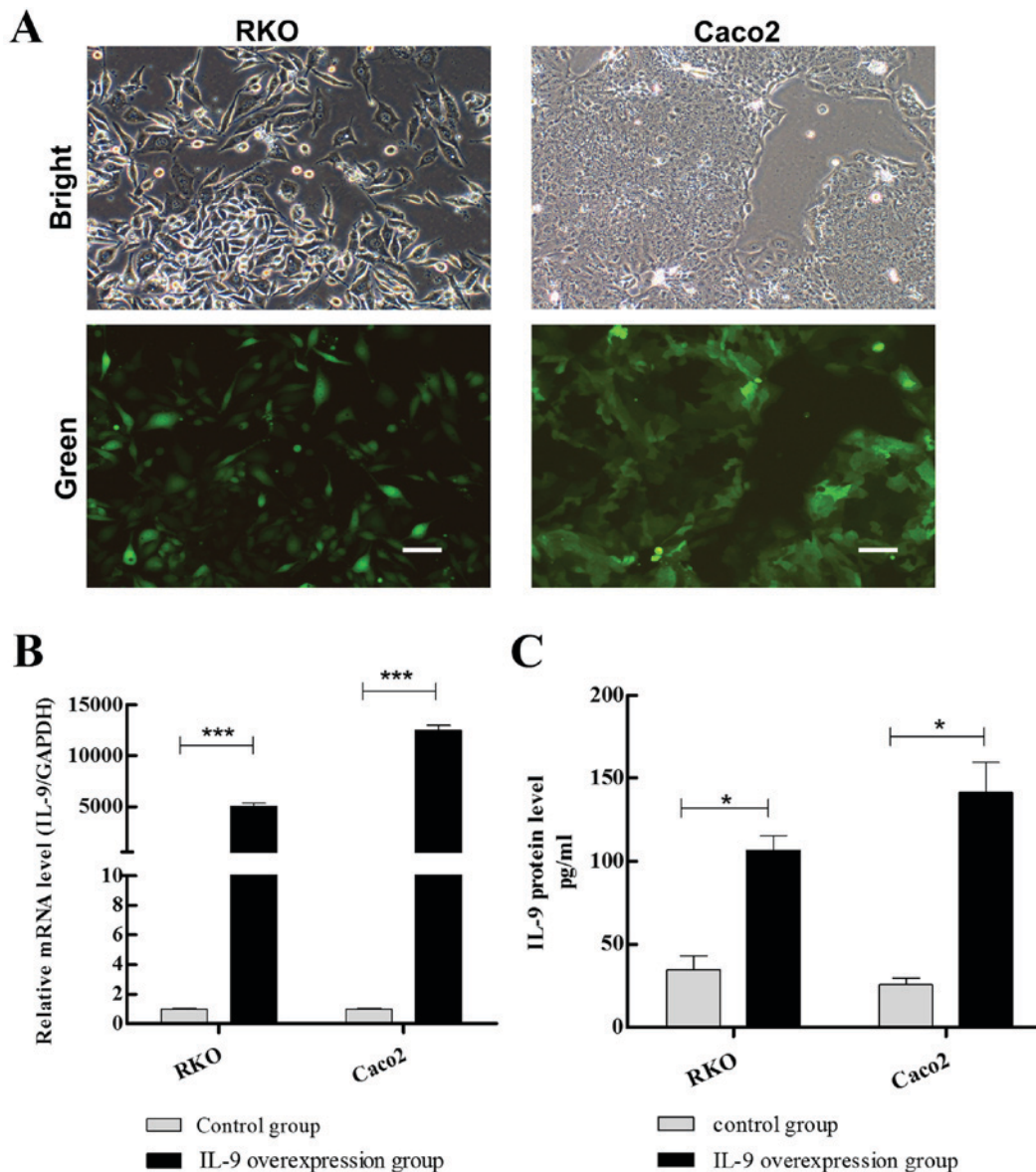


Figure 3. IL9 overexpression in RKO and Caco2 cells. (A) Infection efficiency as determined by light and fluorescence microscopy at 72 h postinfection in RKO and Caco2 cells. Scale bar, 50 μ m. (B) IL-9 mRNA level in RKO and Caco2 cells following overexpression of IL-9. (C) The level of IL-9 protein in the cell culture supernatant following overexpression of IL-9 as determined by ELISA. The level of IL-9 protein was significantly increased following overexpression of IL-9. Data are presented as the mean values \pm standard deviation. * P <0.05 and *** P <0.001. IL-9, interleukin-9.

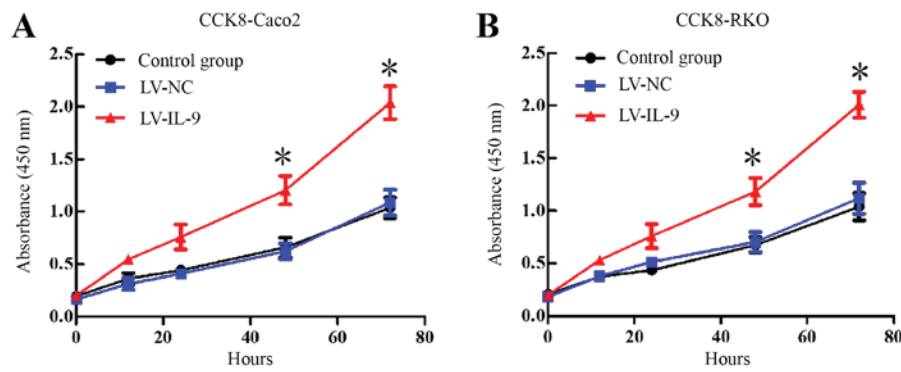


Figure 4. IL-9 promotes RKO and Caco2 cell growth. (A and B) Compared with control group and LV-NC group, overexpression of IL-9 significantly promoted the proliferation of (A) RKO and (B) Caco2 cells. * $P < 0.05$ compared with the control and LV-NC groups. IL-9, interleukin-9; CCK-8, Cell Counting Kit-8; LV, lentiviral vector; NC, negative control.

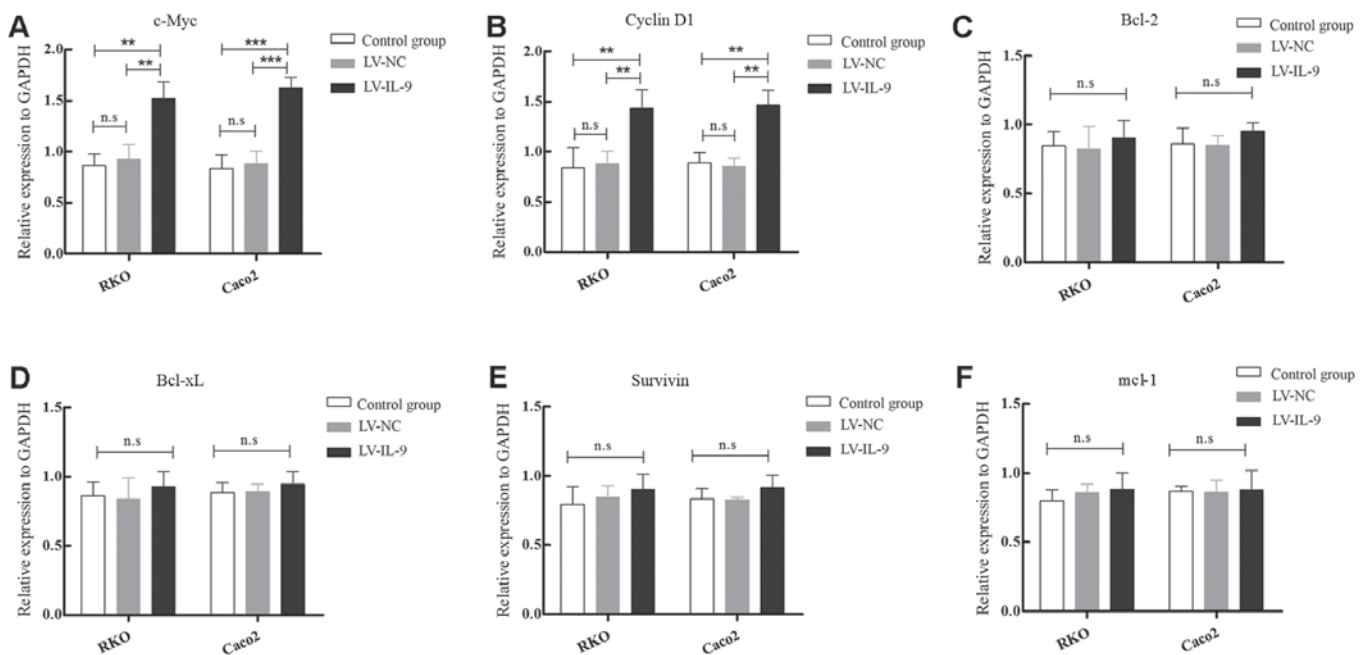


Figure 5. IL-9 upregulates the expression of *c-Myc* and *cyclin D1*. (A and B) A significant increase in the relative expression of (A) *c-Myc* and (B) *cyclin D1* mRNA was observed in the LV-IL-9 group compared with control and LV-NC groups. (C-F) No significant difference was observed in the expression of (C) *Bcl-2*, (D) *Bcl-xL*, (E) *survivin* or (F) *Mcl-1* between the LV-IL-9 and LV-NC groups. ** $P < 0.01$ and *** $P < 0.001$. *c-Myc*, MYC proto-oncogene bHLH transcription factor; *Bcl-2*, B cell lymphoma-2; *Bcl-xL*, Bcl-2-associated X protein; *Mcl-1*, Induced myeloid leukemia cell differentiation protein Mcl-1 homolog; IL-9, interleukin-9; LV, lentiviral vector; NC, negative control.

Discussion

IL-9 is considered as a pleiotropic cytokine that mediates the growth of several types of cells. The role of IL-9 in tumor immunity is controversial, and its involvement in disease remains unclear. Research has confirmed that the OE of IL-9 is associated with the progression of various forms of lymphoma and leukemia (25,26). However, the expression of IL-9 in CAC and its clinical importance remain unclear.

In the current study, the expression of IL-9 in cancer and adjacent colonic mucosa specimens were collected from patients with CAC between 2010 and 2017, and was analyzed using immunohistochemistry. The results confirmed that IL-9 is mainly expressed in intestinal mucosal epithelial cells and inflammatory cell cytoplasm. The expression of IL-9 in cancer tissues was markedly higher than that in adjacent tissues,

which suggests that IL-9 may play an important role in the pathogenesis of CAC. Based on these results, we presume that the expression level of IL-9 fluctuates between cancer types. The objective of our study was to analyze the colonic biopsy specimens from patients with CAC and examine how the expression of IL-9 may relate to the proinflammatory effect of IL-9 in the pathogenesis of UC (27).

To investigate the mechanism of IL-9 function in the pathogenesis of CAC, we constructed lentivirus expressing IL-9 and infected RKO and Caco2 cells to study its role on cell proliferation. The lentiviral vector overexpressing IL-9 gene was successfully constructed and the lentivirus successfully infected RKO and Caco2 cells. IL-9 overexpression could promote the proliferation of colonic epithelial cells by upregulating of the expression of *c-Myc* and *cyclin D1*. In along cancer study, Ye *et al* (28) demonstrated that the proliferation rate of

tumor cells increases with the IL-9 intervention, suggesting that IL-9 promotes the growth of lung cancer cells. The present study also showed that the proliferation of tumor cells increased with the intervention of IL-9, suggesting that IL-9 promotes the growth of colon cancer cells, similar to the findings in lung cancer.

The *c-Myc* gene is typically downregulated in inflammation, and overexpressed in sporadic and colitis-associated colon adenocarcinomas. It regulates cell proliferation or differentiation, and its overexpression is able to induce cell transformation and tumor formation (29). *CyclinD1*, as one of the cell cycle regulatory proteins, is overexpressed in several human tumor types, including non-small cell lung cancer (30), esophageal cancer (31) and head and neck cancer (32). The observations in the present study provided evidence that IL-9 overexpression may upregulate the expression of *c-Myc* and *cyclinD1* in RKO and Caco2 cells as described above. This evidence is in accordance with the results of a previous study (13).

In conclusion, the results of the present study demonstrated that IL-9 promoted the proliferation of RKO and Caco2 colonic epithelial cells, partially via the upregulation of *c-Myc* and *cyclinD1* expression. The results of this study provide an important experimental basis for further study of the role of IL-9 in CAC.

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Availability of data and materials

The datasets generated/analyzed in the present study are available on reasonable request from the corresponding author.

Authors' contributions

LH conceived the study, performed the experimental design and data interpretation, and prepared and revised the manuscript. LT performed the majority of the experiments. YL and RC performed the immunohistochemical assay. PZ and JZ performed the quantitative polymerase chain reaction assay.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the First Hospital of Shanxi Medical University (Taiyuan, China). All patients agreed to participate and signed informed consent forms.

Patient consent for publication

The study participants provided consent for the publication of any associated data/images.

Competing interests

All authors declare that they have no competing interests.

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