

miR-455-5p functions as a potential oncogene by targeting galectin-9 in colon cancer

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Abstract. Although there is evidence that galectin-9 is a critical factor in health and disease, the upstream regulatory microRNA (miRNA or miR) of the protein remains poorly defined. miR-455-5p is characterized as a tumor-associated miRNA in cancer research. However, the actual role of miR-455-5p with respect to inhibiting or promoting tumorigenesis in colon cancer is unclear. The present study aimed to investigate the expression, role and target regulation association of galectin-9 and miR-455-5p in colon cancer. Western blot analysis and reverse transcription-quantitative polymerase chain reaction were used for the detection of the expression levels of galectin-9 and miRNAs. Cell Counting kit-8 test was used for the evaluation of cell proliferation, while flow cytometry was used for cell apoptosis analysis. A potential interaction between galectin-9 and miR-455-5p was predicted by target prediction programs and confirmed by luciferase assay and transfection with miRNA mimics. The present study revealed that elevated expression of galectin-9 and miR-455-5p in colon cancer was associated with HT29 cell proliferation and apoptosis. Furthermore, the present study demonstrated that miR-455-5p reduced galectin-9 expression by directly targeting its 3'-untranslated region. These data suggest that miR-455-5p functions as a potential oncogene in colon cancer by targeting galectin-9.

Introduction

Colon cancer is one of the most common types of gastroenteric tumors for males and females, accounting for a large proportion of cancer-associated mortalities worldwide (1,2). Due to changes in human environment, nutritional habits and life style, the incidence rate of colon cancer has increased worldwide over the last 20 years (3,4). Colon cancer occurs in a multi-step process, and metastasis is the major cause of morbidity and mortality, with ~1/3 of patients with colon cancer ultimately developing metastatic disease (5). The use of chemotherapy and surgical resection for the treatment of malignant colon cancer is increasing, but the results of these treatments are typically poor (6). Therefore, investigation into the molecular mechanism underlying the pathogenesis and progression of colon cancer, and the search for specific, sensitive biomarkers for the early diagnosis and prognosis prediction of colon cancer is required.

There are 11 galectin family members identified in humans at present (7), including galectin-9, which is a type 1 tandem repeat containing a C-terminal coding region determinant (C-CRD) of 149 amino acids and an N-terminal CRD of 148 amino acids (8). The protein was first identified as an eosinophil chemoattractant and activation factor (9,10), and later confirmed as a physiological ligand of T-cell immunoglobulin and mucin domain 3 (11). Galectin-9 is known to exhibit a variety of biological functions, including cell aggregation, adhesion, proliferation, apoptosis and modulation of inflammation (12,13). Attention has previously been focused on the molecular mechanism of galectin-9 in malignant tumors. For example, Nobumoto *et al* (14) confirmed that galectin-9 suppressed tumor metastasis by blocking adhesion to the endothelium and extracellular matrices. A study by Zhang *et al* (15) demonstrated that galectin-9 acted as a prognostic factor with antimetastatic potential in hepatocellular carcinoma. However, the expression and role of galectin-9 in human colon cancer have not been fully verified.

MicroRNAs (miRNAs or miRs) are a class of non-coding single-stranded RNA molecules with ~22-24 nucleotides (16). miRNAs serve a pivotal role in the regulation of target gene expression by binding to the 3'-untranslated regions (3'-UTR) of their target messenger RNA (mRNA), leading to mRNA

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degradation or inhibition of translation into protein (17,18). Currently, >2,042 mature miRNAs have been identified in humans, which constitute a large network that regulates the expression of $\leq 30\%$ of all cellular proteins (19,20). The expression of miRNAs is regulated developmentally and spatially, and increasing evidence has demonstrated that miRNAs modulate a variety of cellular functions, including cell differentiation, proliferation and death (21). Numerous studies have indicated the involvement of miRNAs in the progression and metastasis of numerous types of cancer, suggesting that miRNAs may be used in future therapeutic and diagnostic applications (22-24).

At present, the upstream regulatory miRNA of galectin-9 is undefined. The purpose of the present study was to investigate the upstream regulatory miRNA of galectin-9 in colon cancer. The present study demonstrated that elevated expression levels of galectin-9 and miR-455-5p in colon cancer were associated with HT29 cell proliferation and apoptosis, and confirmed that miR-455-5p directly targets galectin-9 3'-UTR and negatively regulates galectin-9 expression in colon cancer cells.

Materials and methods

Tissue collection. Paired resected surgical specimens from primary tumors and corresponding adjacent non-tumor sites were obtained from 10 patients that underwent primary surgical resection of colon cancer between June and October 2013 at the Department of Gastrointestinal Surgery of Wuhan Union Hospital (Wuhan, China). Tissue specimens were confirmed separately by two experienced pathologists under double-blinded conditions. None of the patients received any therapy prior to operation. The demographic features and clinicopathological data were reviewed in the patients' medical records. The present study was approved by the Ethics Committee of Wuhan Union Hospital and performed with informed consent obtained from all patients. All samples were frozen in liquid nitrogen and stored at -80°C for future molecular analyses.

Cell culture. The human colon cancer HT29 cell line was purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 1 $\mu\text{g}/\text{ml}$ streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 .

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA and miRNA were extracted from the cells and tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) according to the manufacturer's protocol. RNA quantity and quality were determined using 1% agarose gel electrophoresis and an optical density 260/280 absorption ratio of >1.8 using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized using a PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) and a MyCyclerTM thermal cycler (Bio-Rad

Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. The cycling conditions were as follows: 37°C for 15 min, followed by 85°C for 15 sec and then 4°C . RT-qPCR was performed using SYBR[®] Premix Ex TaqTM II (Takara Biotechnology Co., Ltd.) and Mx3000P qPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). Each reaction was performed in a total volume of 20 μl , containing 10 μl SYBR[®] Premix Ex TaqTM II, 2 μl primers, 2 μl template complementary DNA, 0.4 μl ROX Reference Dye (50X; Takara Biotechnology Co., Ltd.) and 5.6 μl distilled H_2O . Cycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles of amplification (95°C for 5 sec, 60°C for 30 sec and 72°C for 30 sec). RT-qPCR was performed in triplicate for each sample. The expression of each type of RNA and miRNA was defined from the quantification cycle (C_q), and relative expression levels were calculated using the $2^{-\Delta\Delta\text{C}_q}$ method (25). Human GAPDH and U6 were used as the housekeeping genes for the amplifications. The PCR primers used in the present study were described previously (26).

Western blot analysis. Proteins were extracted from the cells and tissues using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), and the lysates were cleared by centrifugation at $13,523 \times g$ at 4°C for 15 min. Subsequent to their concentration being measured with the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.), the proteins were mixed with SDS loading buffer, separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Subsequent to the blockage of nonspecific binding sites for 1 h with 5% nonfat milk, the blots were incubated with rabbit anti-human galectin-9 (dilution, 1:1,000; catalog no. YT1841; ImmunoWay Biotechnology Company, Plano, TX, USA) or anti- β -actin (dilution, 1:2,000; catalog no. 12,620; Cell Signaling Technology, Inc., Danvers, MA, USA) primary antibodies at 4°C overnight, followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (dilution, 1:2,000; catalog no. 7074; Cell Signaling Technology, Inc.) at room temperature for 1 h. Proteins were visualized with PierceTM ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). The immunoblots were visualized using ImageJ software version 1.49 (National Institutes of Health, Bethesda, MD, USA).

Flow cytometric analysis for apoptosis. HT29 cells transfected with Galectin-9/pcDNA3.1 vector were seeded in 6-well plates (1×10^6 cells/well) and cultured with DMEM in a humidified chamber at 37°C in 5% CO_2 for 24 h. Cell apoptosis was evaluated using an Annexin-V-FLUOS Staining kit (catalog no. 11858777001; Roche Applied Science, Mannheim, Germany). Briefly, 1×10^6 cells were washed with PBS and centrifuged at $200 \times g$ at room temperature for 5 min. Then, the cell pellets were resuspended in 100 μl Annexin-V-FLUOS labeling solution (containing 2 μl Annexin-V-FLUOS labeling reagent, 2 μl propidium iodide solution and 96 μl incubation buffer) and incubated for 10-15 min at $15-25^{\circ}\text{C}$. Apoptosis was assessed by flow cytometry using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Non-transfected HT29 cells were used as

a negative control. Each group was independently evaluated three times.

Cell proliferation assay. At 24 h post-transfection, the cells were digested using trypsin (Wuhan Amyjet Scientific Co., Ltd., Wuhan, China) and washed twice with PBS (Sangon Biotech Co., Ltd., Shanghai, China), and then seeded into 96-well plates at a concentration of 2×10^3 cells/well. Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assess the cell proliferation activity at 0, 24, 48 and 72 h. A total of 10 μ l CCK-8 was added to each well, and following 2 h of incubation at 37°C, the optical density value at 450 nm was determined with a scan reader (MTX Lab Systems, Inc., Vienna, VA, USA).

Target prediction. miRNAs that target galectin-9 were identified by examining the galectin-9 3'-UTR with bioinformatics algorithms that predict miRNA target sites. Specifically, miRanda (www.microrna.org) and TargetScan (www.targetscan.org) were used for the analysis of the alignment between miRNAs and the 3'-UTR of galectin-9.

Plasmid construction, miRNA synthesis and transfection. The plasmid (p) cytomegalovirus (CMV) -galectin-9-3'-UTR wild-type (WT), pCMV-galectin-9-3'-UTR mutant (MU) and galectin-9 overexpression vector (Galectin-9/pcDNA3.1) were constructed as described previously (26). The specific primers for galectin-9 3'UTR-WT were: Forward, 5'-ATAGAATTC GCGGCTTCCTGGCCCTG-3' and reverse, 5'-CGCAAG CTTTGAATGTGCCACAAGCA-3'. The specific primers for galectin-9 3'UTR-MU were: Forward, 5'-AATGAA AATGCTTGTGGAATTCTCAAAGCTTATCGAT-3' and reverse, 5'-TTTCCAGGAGGGGTGAAGAATTCGTGC ACGGTGCAAGG-3'. miR-455-5p and miR-control (used as a negative control) mimics were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The cells were transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., USA) for 24 h, according to the protocol of the manufacturer.

Luciferase reporter gene assay. The HT29 cells were plated in a 96-well plate and co-transfected with miR-455-5p or control mimics and pCMV-galectin-9 3'-UTR-WT or pCMV-galectin-9 3'-UTR-MU, in addition to the pRL-TK vector (Promega Corporation, Madison, WI, USA), using Lipofectamine 2000. The cells were collected 24 h after transfection, and the luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega Corporation) in a Modulus single-tube multimode reader (Turner BioSystems, Inc.; Promega Corporation). The pRL-TK vector (Promega Corporation, Madison, WI, USA) that provided the constitutive expression of *Renilla luciferase* was co-transfected as an internal control to correct for differences between transfection and harvest efficiencies. The transfections were performed at least twice in independent experiments.

Statistical analysis. The Student's *t*-test was used to evaluate statistical significance. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were repeated >3 times, and the results from a representative experiment were

selected to draw diagrams and data analysis. All data were statistically analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Expression and role of galectin-9 in colon cancer tissue and in colon cancer cell proliferation and apoptosis. The expression levels of galectin-9 in colon cancer tissues and the corresponding adjacent tissues from 10 patients were determined using RT-qPCR and western blot analysis. Galectin-9 was revealed to be significantly downregulated in colon cancer tissue at the mRNA and protein level compared with the corresponding adjacent tissue ($P=0.0405$ and $P=0.0037$; Fig. 1A and B, respectively).

To investigate the role of galectin-9 in colon cancer cell apoptosis, flow cytometric analysis was evaluated using an Annexin-V-FLUOS Staining kit. The present study revealed that overexpression of galectin-9 promoted HT29 cell apoptosis (Fig. 1C).

In order to additionally examine the role of galectin-9 in colon cancer cell proliferation, the present study constructed the galectin-9 overexpression vector Galectin-9/pcDNA3.1 and transfected it into HT29 cells. CCK-8 assay revealed that overexpression of galectin-9 inhibited HT29 cell proliferation (Fig. 1D).

Expression of candidate miRNAs and role of miR-455-5p in colon cancer. The target prediction programs miRanda and TargetScan were used to predict and identify miRNAs that may target galectin-9. The present study identified four miRNAs (miR-22, 296-3p, 455-5p and 491-5p) that were potential regulators of galectin-9 (26).

The expression of the aforementioned four miRNAs in colon cancer tissues and corresponding adjacent tissues was determined. The results indicated that the expression of miR-22 was significantly downregulated in colon cancer tissue compared with the corresponding adjacent tissue ($P=0.0397$; Fig. 2A), while miR-455-5p was significantly upregulated in colon cancer tissue compared with the corresponding adjacent tissue ($P=0.0346$; Fig. 2C). miR-296-3p and miR-491-5p did not exhibit a significant difference in expression between colon cancer tissue and the corresponding adjacent tissue ($P=0.9063$ and $P=0.9477$; Fig. 2B and D, respectively). miR-455-5p was selected in the present study for further experiments, as its expression level exhibited an inverse correlation with galectin-9 expression.

The present study subsequently examined the role of miR-455-5p in colon cancer cell apoptosis and proliferation, revealing that miR-455-5p inhibited HT29 cell apoptosis (Fig. 3A) and promoted HT29 cell proliferation (Fig. 3B).

miR-455-5p directly targets and downregulates galectin-9 in HT29 cells. To validate whether galectin-9 was a direct target of miR-455-5p, the present study constructed galectin-9 3'-UTR-WT and galectin-9 3'-UTR-MU plasmids (Fig. 4A), and transfected them into HT29 cells with miR-control or miR-455-5p mimic, respectively. Luciferase activity was measured 24 h after transfection. The luciferase activity in the cells co-transfected with miR-455-5p mimic

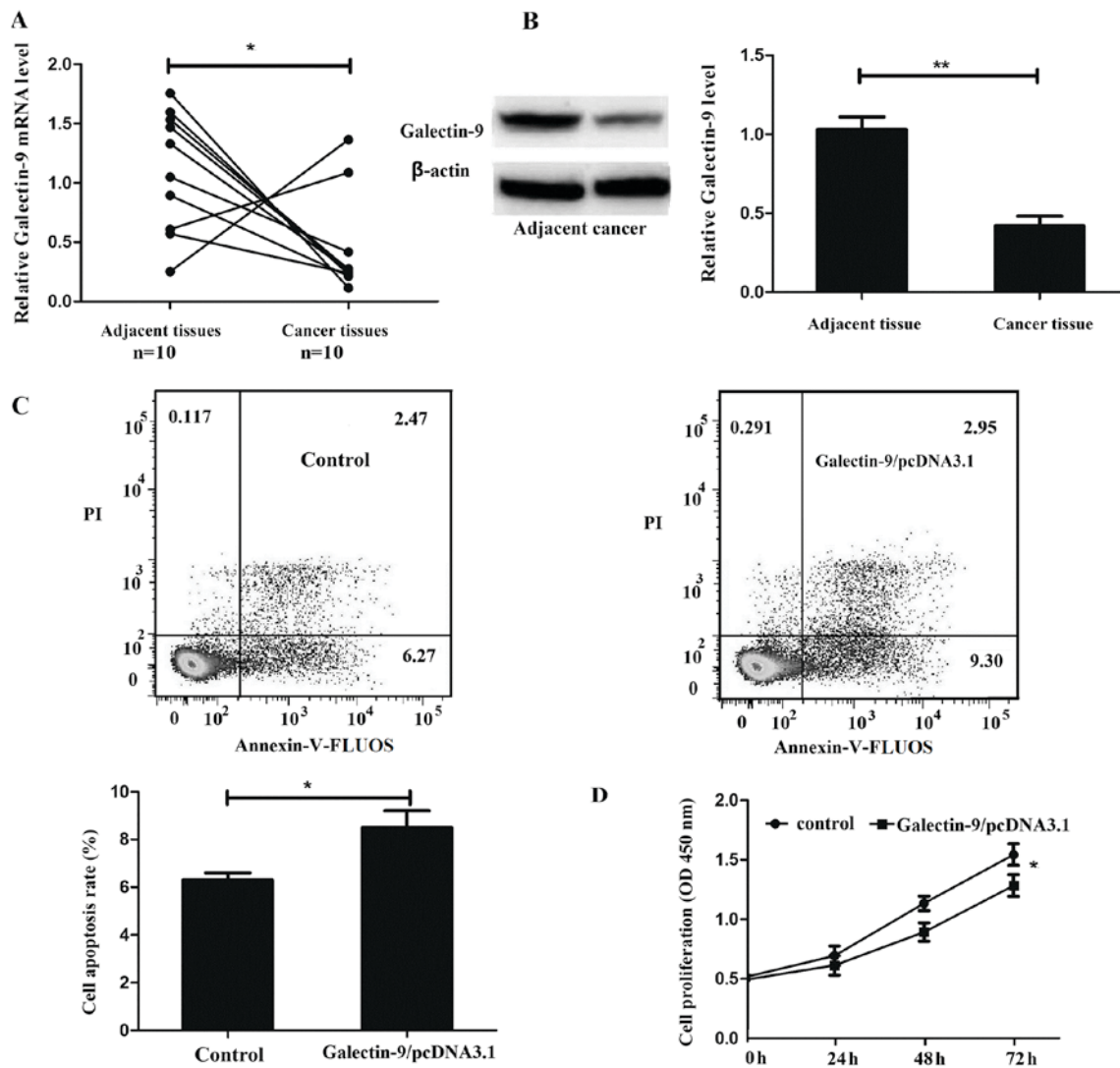


Figure 1. Expression of galectin-9 in colon cancer tissue, and its role in cell apoptosis and proliferation. (A) Galectin-9 mRNA level was significantly downregulated in colon cancer tissues compared with that in the corresponding adjacent tissues, n=10. (B) Galectin-9 protein level was significantly downregulated in colon cancer tissues compared with that in the corresponding adjacent tissues, n=10. (C) Overexpression of galectin-9 promoted HT29 cell apoptosis. (D) Overexpression of galectin-9 inhibited cell proliferation. *P<0.05, **P<0.01. mRNA, messenger RNA; OD, optical density; PI, propidium iodide.

and galectin-9 3'-UTR-WT plasmid significantly decreased compared with that in cells co-transfected with miR-control mimic or galectin-9 3'-UTR-MU plasmid (Fig. 4B).

To investigate the potential correlation between miR-455-5p and galectin-9 mRNA/protein expression in colon cancer cells, miR-control or miR-455-5p mimic were transfected into HT29 cells, and the mRNA/protein expression levels of galectin-9 were examined by RT-qPCR and western blot analysis, respectively. The expression of galectin-9 mRNA (P=0.0043; Fig. 4C) and protein (P=0.0027; Fig. 4D) were observed to be significantly downregulated subsequent to transfection with miR-22 mimics, compared with the results obtained for the miR-control. These findings indicate that galectin-9 is a direct downstream target of miR-455-5p in HT29 cells.

Discussion

Galectin-9 has been detected extracellularly and intracellularly, and the expression of the protein is widely distributed in tissues (27,28). Indeed, the available results suggest that

galectin-9 expression is frequently altered when comparing tumor tissue with normal tissue (29). In addition, one study supports the hypothesis that galectin-9 is involved in several aspects of tumor progression (30). Galectin-9 also induces the apoptosis of various cell types, including human melanoma, T cell and leukemia cell lines (13,31,32). The present study explored the expression and potential role of galectin-9 in colon cancer, and noticed that galectin-9 was significantly downregulated in colon cancer tissue compared with corresponding adjacent tissue. Furthermore, overexpression of galectin-9 inhibited HT29 cell proliferation and promoted HT29 cell apoptosis. These results suggest that galectin-9 is important in colon cancer.

Numerous studies highlight the impact of miRNA on the tumorigenesis of human carcinoma (33-35). To define the upstream regulatory miRNA of galectin-9, the target prediction programs miRanda and TargetScan were used to predict miRNAs that possibly target galectin-9. miR-22, 296-3p, 455-5p and 491-5p were identified as potential miRNAs, and their expression levels were measured in colon cancer

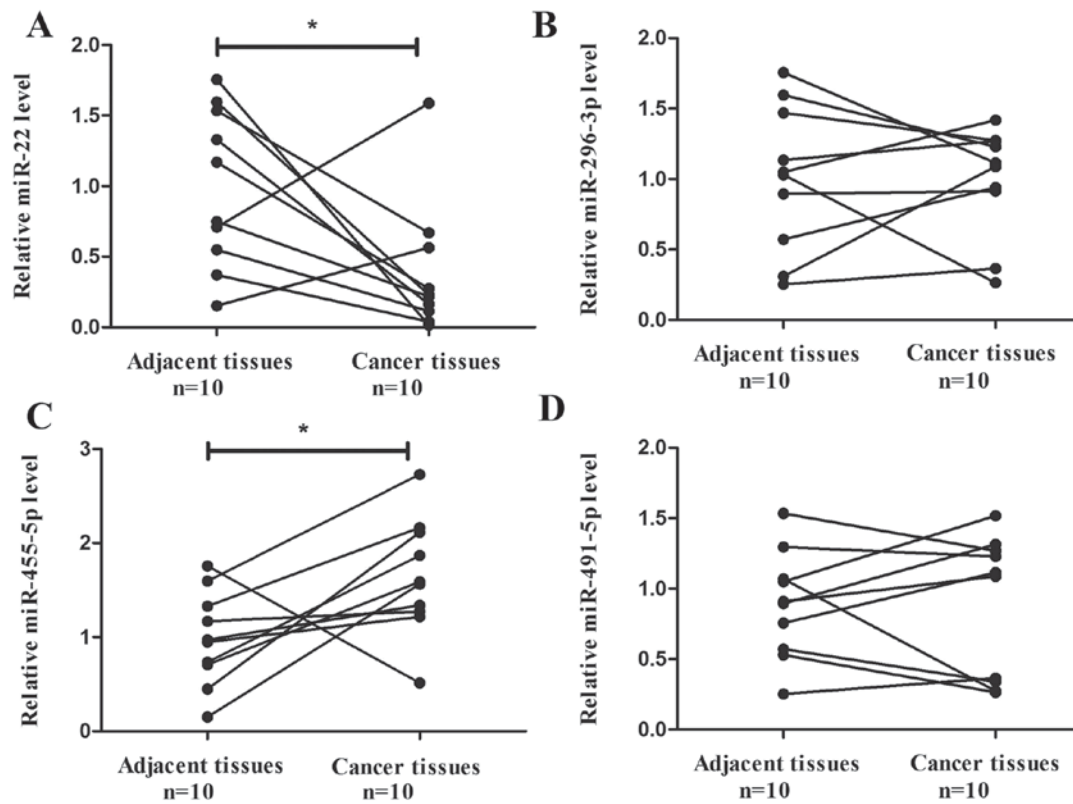


Figure 2. Expression levels of four miRNAs that may target galectin-9. (A) miR-22 was downregulated in colon cancer tissue compared with corresponding adjacent tissue, n=10. (B) Increasing the level of miR-296-3p expression did not result in a significant difference between tumor and corresponding adjacent tissues. (C) miR-455-5p was significantly upregulated in colon cancer tissue compared with corresponding adjacent tissue, n=10. (D) Increasing the level of miR-491-5p expression did not result in a significant difference between tumor and corresponding adjacent tissues. *P<0.05. miR, microRNA.

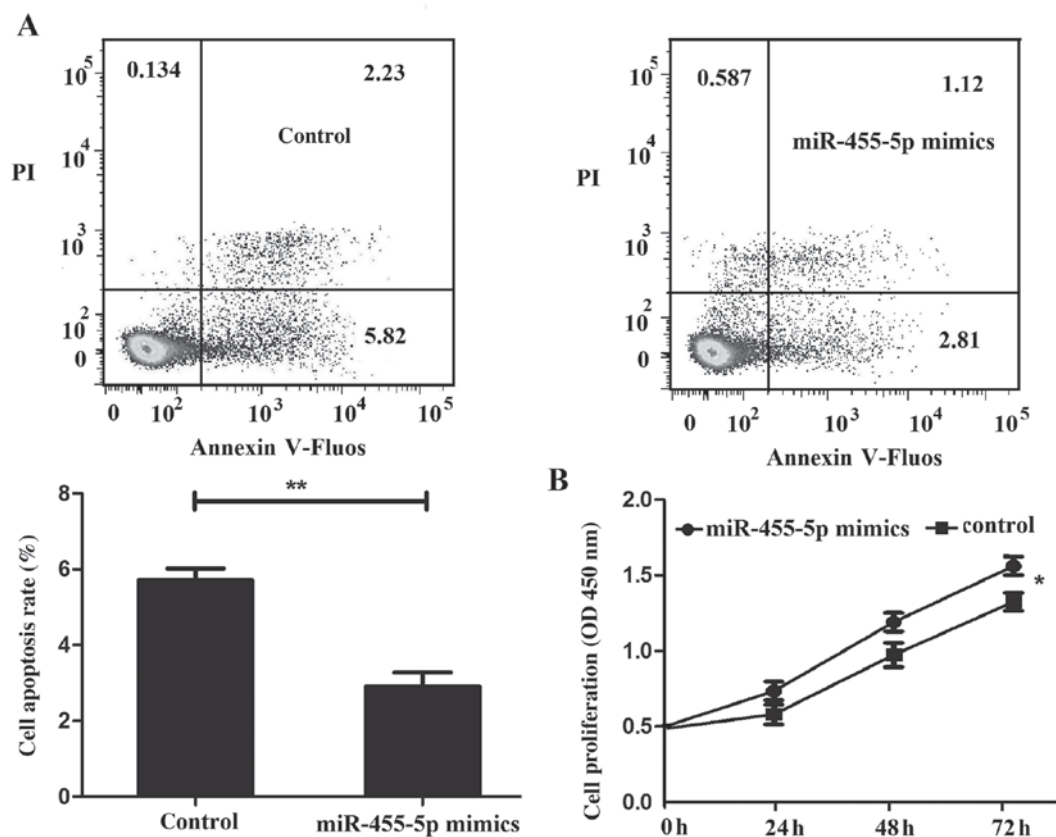


Figure 3. Role of miR-455-5p in colon cancer cell apoptosis and proliferation. (A) miR-455-5p inhibited HT29 cell apoptosis. (B) miR-455-5p promoted HT29 cell proliferation. *P<0.05, **P<0.01. miR, microRNA; PI, propidium iodide.

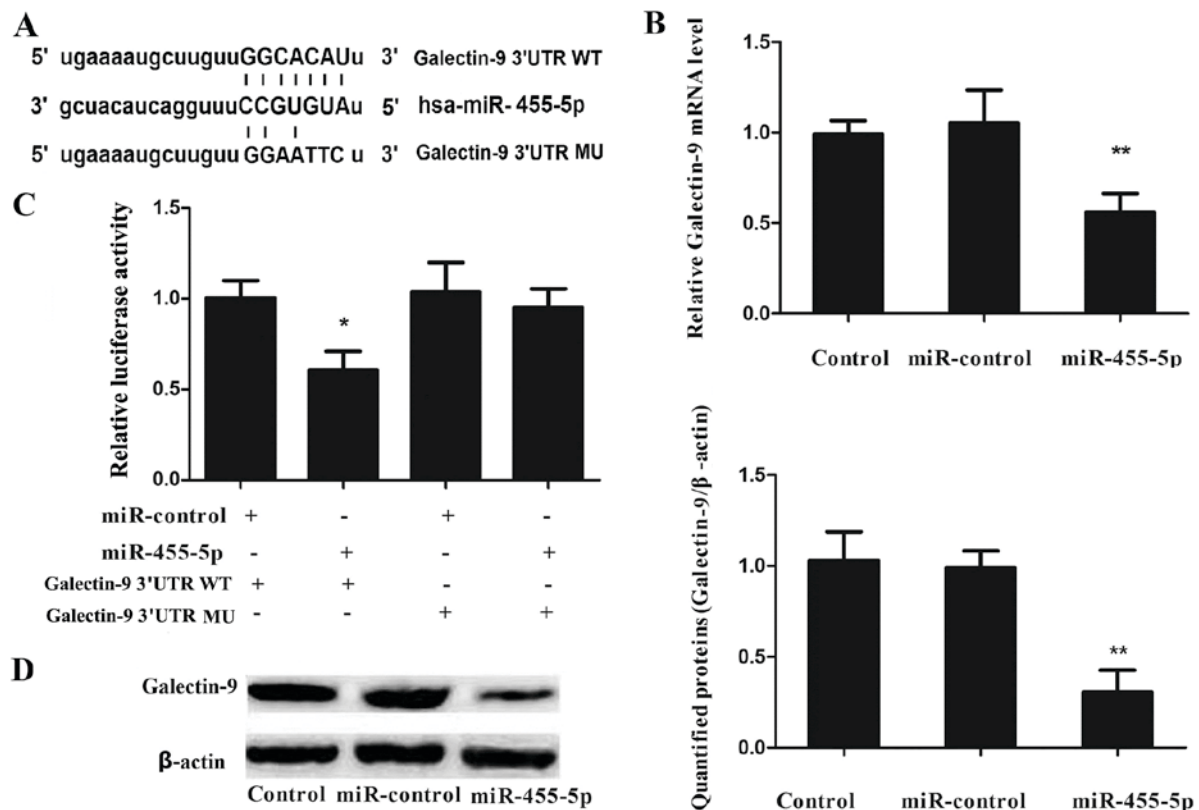


Figure 4. miR-455-5p directly targets and downregulates galectin-9 in HT29 cells. (A) Conserved or mutated forms of the miR-455-5p binding sequence were inserted into the luciferase gene to generate galectin-9 3'-UTR-WT or galectin-9 3'-UTR-MU plasmids, respectively. (B) Expression of galectin-9 mRNA was significantly downregulated following transfection with a miR-455-5p mimic. (C) miR-455-5p suppressed luciferase reporter gene activity in HT29 cells. (D) The expression of galectin-9 protein was significantly downregulated subsequent to transfection with miR-455-5p mimics. * $P < 0.05$, ** $P < 0.01$. UTR, untranslated region; WT, wild type; MU, mutant; mRNA, messenger RNA; miR, microRNA; hsa, *Homo sapiens*.

and corresponding adjacent tissues. Of these four miRNAs, miR-455-5p was significantly upregulated, while miR-22 was downregulated, in colon cancer tissue compared with the corresponding adjacent tissue. By contrast, miR-296-3p and 491-5p did not exhibit a significant difference. miR-455-5p has recently been shown to be important in the progression of numerous types of malignancy. Liu *et al* (36) identified that miR-455-5p was associated with anaplastic lymphoma kinase expression. Shoshan *et al* (37) confirmed that miR-455-5p contributes to melanoma growth and metastasis through the downregulation of the tumor-suppressor gene cytoplasmic polyadenylation element binding protein 1. However, the exact role with respect to influencing cell proliferation and apoptosis, and the regulatory mechanism of miR-455-5p in colon cancer remains unclear. The present study demonstrated that miR-455-5p promoted HT29 cell proliferation and inhibited HT29 cell apoptosis. These results suggested that miR-455-5p serves an oncogenic role in colon cancer. In addition, miR-455-5p and galectin-9 expression exhibited an inverse correlation and role in influencing cell proliferation and apoptosis, which provides a foundation for additional investigation into their association.

To explore whether miR-455-5p is involved in the regulation of galectin-9 expression, a Dual-Luciferase Reporter Assay System was employed. The luciferase reporter assay indicated that the luciferase activity of the reporter containing the wide-type 3'-UTR of the galectin-9 gene

decreased following treatment with miR-455-5p mimic, indicating that miR-455-5p suppresses gene expression through miR-455-5p-binding sequences at the 3'-UTR of galectin-9. In addition, RT-qPCR and western blot analysis revealed that the mRNA and protein expression of galectin-9 was inhibited by treatment with the miR-455-5p mimic in HT29 cells. These data suggest that miR-455-5p reduces galectin-9 expression by inhibiting translation and/or causing mRNA instability.

In summary, the present study provides evidence that miR-455-5p mediates the downregulation of galectin-9 in colon cancer. The present study demonstrated that miR-455-5p promoted HT29 cell proliferation and inhibited HT29 cell apoptosis by suppressing galectin-9 expression. miRNA-455-5p functions as a potential oncogene in colon cancer, and the miRNA-455-5p/galectin-9 axis provides a novel insight into the pathogenesis of colon cancer.

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