

miRNA-766 induces apoptosis of human colon cancer cells through the p53/Bax signaling pathway by MDM4

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Abstract. miRNAs are closely associated with tumor genesis and development. The present study investigated the role of the expression of miRNA-766 in the survival of patients with colon cancer and the underlying molecular mechanisms. Reverse transcription-quantitative polymerase chain reaction analysis and microarray analysis were used to analyze the expression of miRNA-766. The results revealed that the expression of miRNA-766 was decreased in patients with colon cancer. The overall survival and disease-free survival rates of patients with colon cancer with a high expression of miRNA-766 were prolonged, compared with those with a low expression of miRNA-766. The overexpression of miRNA-766 reduced cell growth and induced apoptosis in colon cancer cells through suppression of the MDM4/p53 pathway. By contrast, the downregulation of miRNA-766 promoted cell growth and reduced apoptosis in colon cancer cells through activation of the MDM4/p53 pathway. The promotion of MDM4 attenuated the anticancer effect of miRNA-766 in colon cancer cells. These results demonstrated that miRNA-766 induced cell apoptosis in human colon cancer through MDM4/p53.

Introduction

Colon cancer is one of the most common malignancies severely threatening human health (1). It ranks third highest worldwide in terms of cancer-associated mortality rates (1). According to statistics, there were ~1,000,000 new cases of colon cancer in 2008 worldwide, and ~500,000 individuals succumbed to mortality (2). The morbidity rate of colon cancer in China has exhibited an increasing trend in recent years. This can

be ascribed to improvements in nationwide living standards, changes in dietary patterns, aging of the population, and rapid developments in endoscopy (3,4). Therefore, colon cancer has become a serious threat to human health as one of the most common malignancies (3,4).

Colon cancer has a high mortality rate regardless of the advances in surgical technology and other treatments and despite novel molecular preparations emerging in previous years (5). This may be attributed mainly to the frequent recurrence and metastasis of colon cancer (5). Therefore, early diagnosis and treatment is of significance for improving prognosis of patients with colon cancer (5). At present, the mechanism underlying its tumorigenesis remains to be fully elucidated. Understanding its corresponding mechanism is important to develop novel strategies and identify novel target genes for effectively treating colon cancer (6).

MicroRNAs are types of small non-coding RNA molecule of ~19-24 nucleotides in length (7). They are relatively conserved during biological evolution and do not encode protein. However, they can regulate target genes through directly binding with the 3'untranslated region (3'-UTR) of target mRNA (8).

It has been demonstrated in previous years that miRNAs are closely associated with tumor genesis and development. Compared with normal tissue, the miRNA expression profile in tumor tissue is significantly altered. Furthermore, miRNAs interact with traditional tumor suppressor genes and oncogenes (9). Therefore, they have dual-identity during tumorigenesis and development. It is estimated that >50% of miRNA upstream regulatory genes locate in tumor-associated regions in chromosomes. There are a variety of miRNAs, including those functioning as oncogenes and others as tumor suppressor genes. miRNAs can exert oncogenic functions, inhibit the expression of tumor suppressor genes, and provide favorable conditions for tumor proliferation, migration, metastasis and infiltration (10). By contrast, they can function as a tumor suppressor gene, inhibit expression of oncogene, and contribute to tumor cell apoptosis, differentiation and treatment (11). The earliest report regarding the correlation between miRNA and tumors was of the 13q14 gene deletion in chronic lymphocytic leukemia (11). The oncogene miRNA17-92' was the first miRNA identified in mammals (11); it shows abnormally increased expression in lung cancer and lymphoma. Mutation in relevant miRNAs

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can activate the expression of its oncogene or induce loss or changes in a tumor suppressor gene, which results in tumor genesis and development (11).

MDM4 is a type of proto-oncogene locating in chromosome 1q23, which encodes 11 exons and 490 amino acids (8). The MDM4 protein structure includes a p53-binding domain (p53 BD) locating in the N-terminal, and an amino acid region locating in the RING finger domain and center (8). The RING finger domain is the major determinant of MDM4 and MDM2 acting on p53 (8), and a heterodimer can be formed through the domain (8). MDM4 binds with p53 through p53BD in its N-terminal, and inhibits the transcriptional activity of p53. It has been verified in a previous study that MDM4 is partly correlated with tumors (12). For example, the overexpression of MDM4 can be detected in certain human tumor cell lines, and MDM4 proliferation has been found in solid tumors, including breast cancer and colon cancer (7,12). Therefore, it is hypothesized that MDM4 in human tumors can promote tumor genesis or progression. The present study investigated whether the role of the expression of miRNA-766 affects human colon cancer survival rate and examined the underlying molecular mechanisms.

Materials and methods

Study population. A total of 102 patients with human colon cancer and 57 normal volunteers were recruited from the Second Affiliated Hospital, Shantou University Medical College (Shantou, China) from March 2012 to July 2012. Colon cancer tissue samples were acquired in patients undergoing surgery, and para-carcinoma tissues were collected at a distance of 5 cm from the cancer tissue samples. The present study was approved by the Ethical Agent Will of the Second Affiliated Hospital, Shantou University Medical College.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated using a TRIzol-based (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) RNA isolation protocol and 500 ng RNA was reverse transcribed using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR analysis was performed using SYBR-Green PCR Master Mix (PE Applied Biosystems; Thermo Fisher Scientific, Inc.) with the Applied Biosystems 7900HT real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: Hot start at 94°C for 10 min; 40 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C, and 10 min at 72°C. miR-766 forward, 5'-TCGAGTACTTGAGATGGAGTTTTT-3' and reverse, 5'-GGCCGCGTTGCAGTGAGCCGAG-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACG AATTGCGTGCAT-3'. miRNA expression was measured using $2^{-\Delta\Delta Cq}$ (13).

Microarray analysis. RNA cleanup was performed using the RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA). Total RNA was reverse transcribed into cDNA using an oligo d(T) (Bio-Rad Laboratories, Inc., Hercules, CA, USA). cRNA was hybridized onto the Affymetrix Human

Table I. Distribution of selected characteristics among patients with colon cancer and controls.

Characteristic	Colon cancer, n (%)	Control, n (%)	P-value
Number of individuals	102	57	
Age (years)			
<60	57 (55.88)	31 (54.39)	
≥60	45 (44.12)	26 (45.61)	
Sex			
Male	78 (76.47)	46 (80.70)	
Female	24 (23.53)	11 (19.30)	
Smoking status			0.009
No	41 (40.20)	31 (54.39)	
Yes	61 (59.80)	26 (45.61)	
Drinking status			<0.001
No	37 (36.27)	33 (57.89)	
Yes	65 (63.73)	24 (42.11)	

Genome U133 Plus 2.0 Array, staining was performed with a Fluidic Station-450 and GeneChips were scanned with the Affymetrix GeneChip Scanner 7G. Data were quantified and feature-extracted using Agilent Feature Extraction software (version A.10.7.3.1; Agilent Technologies, Inc., Santa Clara, CA, USA).

Cell culture and cell transfection experiments. Human Caco2 cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's minimal essential medium (DMEM; GE Healthcare Life Sciences, Logan, UT, USA), 10% fetal bovine serum (FBS; GE Healthcare Life Sciences) at 37°C in 5% CO₂ and 95% relative humidity. miRNA (miR)-766 mimics and negative control mimics were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Subsequently, 20 ng/ml miR-766, anti-miR-766 mimics and negative control mimics were transfected into Caco2 cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Cell viability assay and analysis of apoptosis. The cells (1x10⁶ cell/ml) were incubated with medium containing MTT (5 mg/ml) for 4 h and dissolved with 150 μ l DMSO. The medium was removed and dissolved with 150 μ l DMSO for 15 min at room temperature. The absorbance was measured at 490 nm using a microplate reader.

To analyze apoptosis, the cells were washed twice with ice-cold PBS and resuspended in 500 μ l binding buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Subsequently, 5 μ l annexin V-fluorescein isothiocyanate and 5 μ l propidium iodide (both Nanjing KeyGen Biotech Co., Ltd.) were added and the cells were incubated for 15 min at room temperature in the dark. Apoptosis was analyzed using a FACSsort flow cytometer and quantified using BD CellQuest™ Pro software (BD Biosciences, Franklin Lakes, NJ, USA).

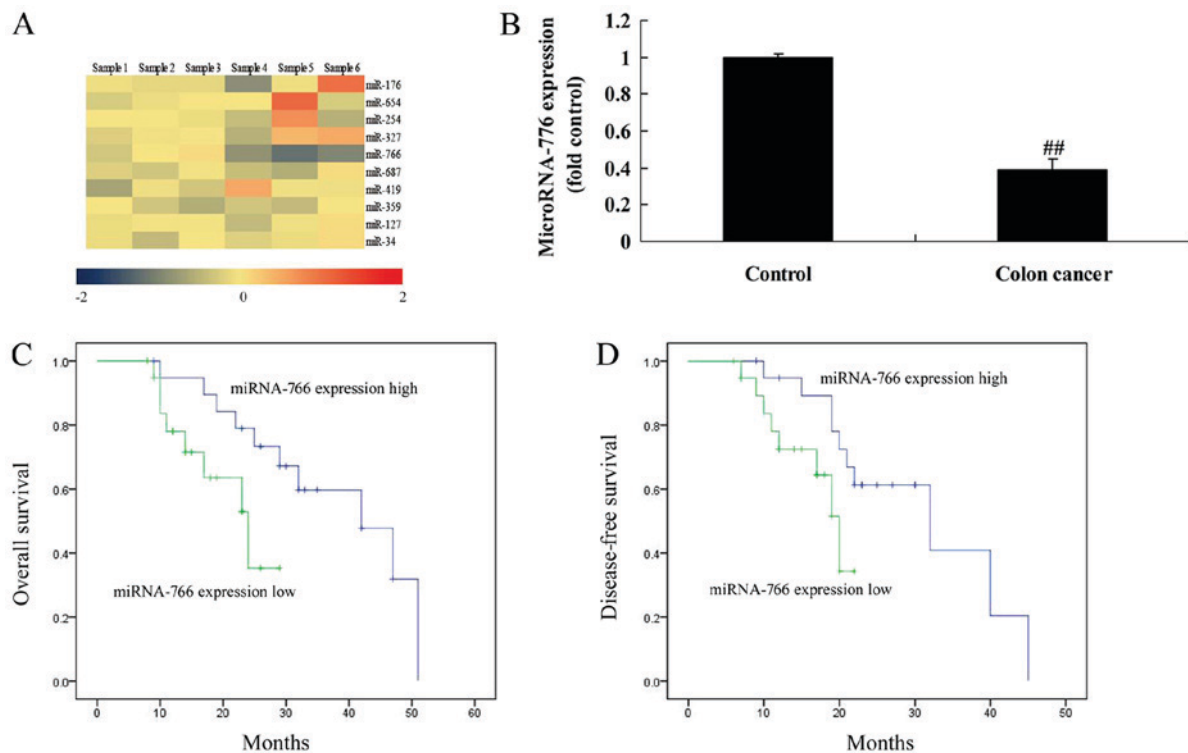


Figure 1. Expression of miR-766 in patients with colon cancer. (A) Heat map and (B) reverse transcription-quantitative polymerase chain reaction analyses of the expression of miR-766 in patients with colon cancer. (C) Overall and (D) disease-free survival rates of patients with colon cancer with differing expression levels of miR-766. ## $P < 0.01$ vs. Control. Control, 57 normal volunteers; Colon cancer, 102 patients with colon cancer; miR/miRNA, microRNA.

Cell migration assay. The Caco2 cells (1×10^5 cell/ml) were seeded on 24-well plates and were added to the upper chamber of each migration well (Corning Corporation, Corning, NY, USA). DMEM (500 μ l) with 20% FBS was added to the lower chamber and incubated for 48 h at 37°C. The lower side were fixed with 75% ice-alcohol for 30 min and stained with 1% crystal violet solution for 1 h at room temperature. The cells were counted under a fluorescence microscope (Axio version II, Carl Zeiss AG, Oberkochen, Germany).

Western blot analysis. Total protein was extracted in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) on ice for 30 min and then centrifuged at 12,000 \times g for 10 min at 4°C. Total protein was quantified using a BCA assay (Beyotime Institute of Biotechnology) and total protein samples (50 μ g) were subjected to 8-12% SDS-polyacrylamide gel electrophoresis and then transferred onto PVDF membranes. The membranes were blocked with 5% skimmed milk in TBST, and incubated at 4°C overnight with the following primary antibodies: MDM4 (cat. no. sc-14738; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), B-cell lymphoma 2-associated X protein (Bax; cat. no. sc-6236, 1:1,000), p53 (cat. no. sc-47698; 1:1,000) and GAPDH (cat. no. sc-51631; 1:5,000; all Santa Cruz Biotechnology). The membranes were washed with TBST for 15 min and then incubated with the anti-rabbit horseradish peroxidase secondary antibody (cat. no. sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at 37°C. The protein bands were detected with enhanced chemiluminescence (Beyotime Institute of Biotechnology).

Analysis of caspase-3/9 activity levels. Total protein was extracted using RIPA buffer (Beyotime Institute of Biotechnology) on ice for 30 min and then centrifuged at 12,000 \times g for 10 min at 4°C. Total protein was quantified using a BCA assay (Beyotime Institute of Biotechnology) and total protein samples (10 μ g) were used to measure the caspase-3/9 activity levels using caspase-3/9 activity kits. The absorbance was measured at 405 nm using a microplate reader.

Statistical analysis. The data are expressed as the mean \pm standard deviation. All statistical analyses were performed using SPSS version 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical comparisons of >2 or three groups were performed using Student's t-test or a one-way analysis of variance, followed by a Bonferroni post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miRNA-766 in patients with colon cancer. Firstly, the present study analyzed changes in the expression of miRNAs in patients with colon cancer. As shown in Fig. 1A and B, the expression of miRNA-766 in patients with colon cancer was elevated, compared with that in the control group. The overall survival (OS) and disease-free survival (DFS) rates of patients with colon cancer with a high expression of miRNA-766 were prolonged, compared with those with a low expression of miRNA-766 (Fig. 1C and D). The baseline characteristics of the 102 patients with colon cancer and 57 normal volunteers are shown in Table I.

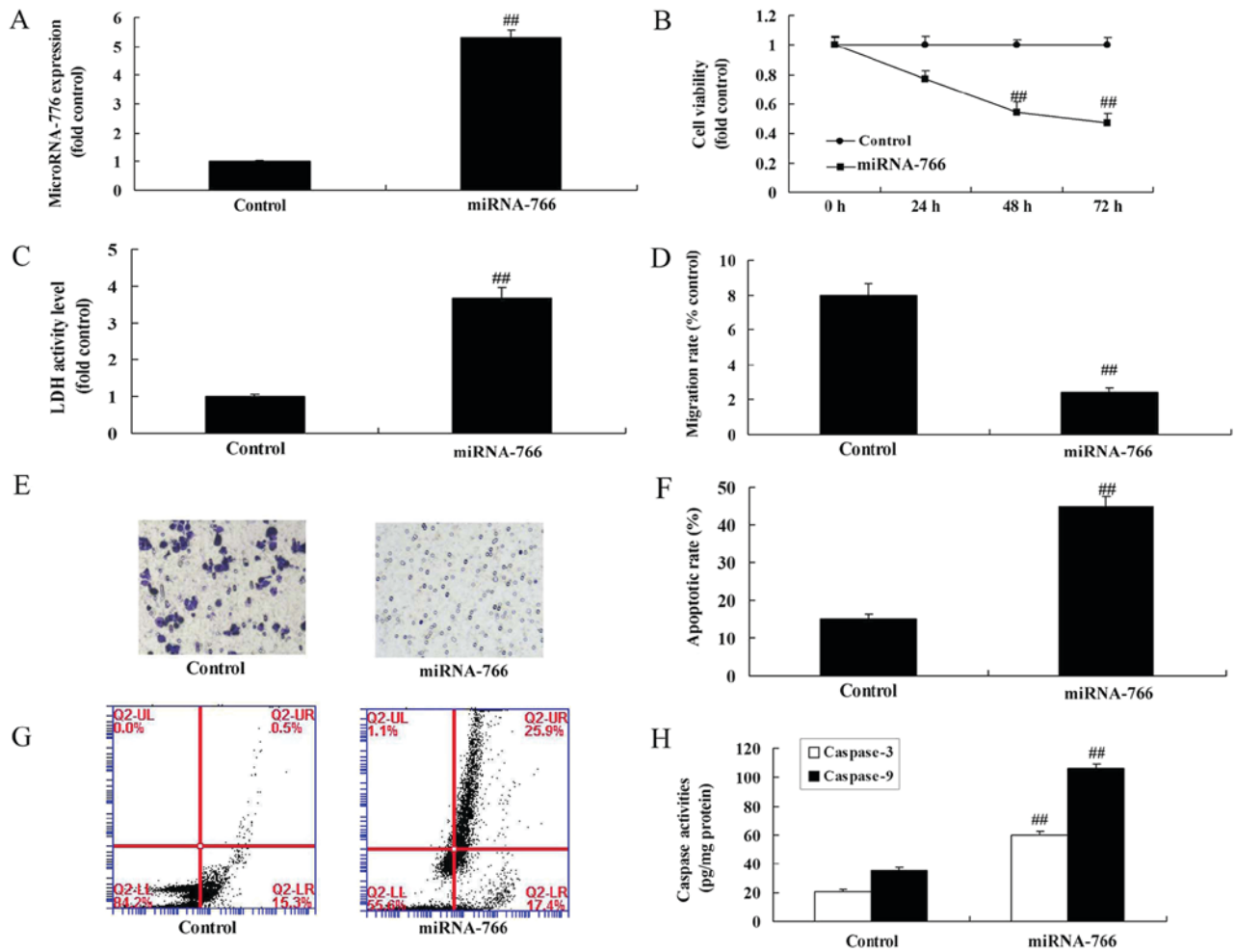


Figure 2. Overexpression of miRNA-766 regulates cell growth of colon cancer cells. (A) Expression of miRNA-766. (B) Cell growth. (C) LDH activity. (D) Cell migration rates and (E) images. Magnification, x100. (F) Apoptotic rates quantified from (G) flow cytometry. (H) Caspase-3/9 activity levels. ## $P < 0.01$, vs. Control. Control, negative control group; miRNA-766, overexpression of miRNA-766 group; miRNA, microRNA; LDH, lactate dehydrogenase.

miRNA-766 regulates cell growth of colon cancer cells. To examine the function of miRNA-766 in the cell growth of colon cancer cells, the present study analyzed the effects of miRNA-766 or anti-miRNA-766 on the growth of colon cancer cells. As shown in Fig. 2A, the expression of miRNA-766 was increased using miRNA-766 mimics in Caco2 cells, compared with that in the negative control group. The overexpression of miRNA-766 reduced cell growth and migration, and promoted lactate dehydrogenase (LDH) activity, apoptotic rate and caspase-3/9 activity levels in the Caco2 cells, compared with cells in the negative control group (Fig. 2B-H). Subsequently, it was confirmed that the expression of miRNA-766 was inhibited in Caco2 cells by using anti-miRNA-766 mimics, compared with expression in the negative control group (Fig. 3A). The downregulation of miRNA-766 promoted cell growth and migration, and reduced the LDH activity, apoptotic rate and caspase-3/9 activity levels in Caco2 cells, compared with cells in the negative control group (Fig. 3B-H).

miRNA-766 regulates the MDM4/p53 pathway in colon cancer cells. Subsequently, the present study examined the mechanism underlying the effect of miRNA-766 on colon

cancer cell growth. As shown in Fig. 4A and B, the putative miR-766-binding sequence in the 3'UTR of MDM4 mRNA and luciferase activity was attenuated following the overexpression of miRNA-766, compared with the negative control group. However, the overexpression of miRNA-766 suppressed the protein expression of MDM4, and induced that of p53 and Bax in the Caco2 cells, compared with the negative control group (Fig. 4C-F). The downregulation of miRNA-766 induced the protein expression of MDM4, and suppressed that of p53 and Bax in Caco2 cells, compared with the negative control group (Fig. 4G-J).

Promotion of MDM4 attenuates the anticancer effect of miRNA-766 in colon cancer cells. To further assess the relevance of the miR-766/MDM4 interaction in p53 signaling, an MDM4 plasmid was utilized to induce the expression of MDM4 in Caco2 cells overexpressing miR-766. As shown in Fig. 5A-D, the MDM4 plasmid induced the protein expression of MDM4, and suppressed that of p53 and Bax in Caco2 cells overexpressing miR-766, compared with the cells overexpressing miR-766 without the plasmid. The overexpression of MDM4 promoted cell growth and migration, and reduced LDH activity, apoptotic rate and caspase-3/9 activity levels in

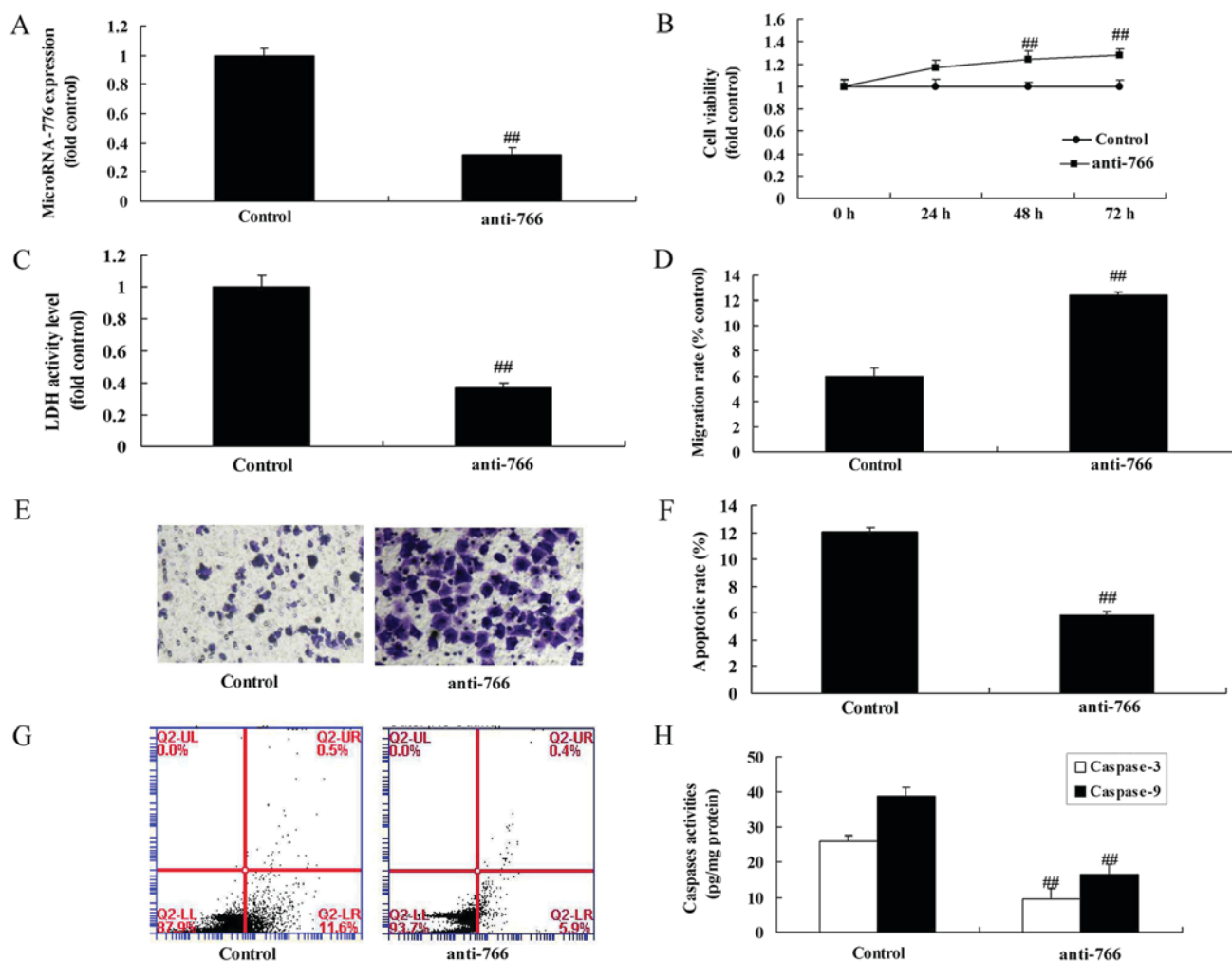


Figure 3. Downregulation of miRNA-766 regulates the growth of colon cancer cells. (A) Expression of miRNA-766. (B) Cell growth. (C) LDH activity. (D) Cell migration rate and (E) images. Magnification, $\times 100$. (F) Apoptotic rate quantified from (G) flow cytometry. (H) Caspase-3/9 activity levels. $^{##}P < 0.01$, vs. Control. Control, negative control group; anti-766, downregulation of miRNA-766 group; anti-766, downregulation of miRNA-766 group; miRNA, microRNA; LDH, lactate dehydrogenase.

the Caco2 cells overexpressing miR-766, compared with the negative control group (Fig. 5E-K).

Discussion

Colon cancer has a complicated pathogenic process (14). It is controlled by multiple genes, has different stages, and is formed over a long period (14). Early diagnosis, together with tumor recurrence monitoring and effective development of novel treatments, is important for patients with colon cancer (13). It has been found in previous years that several miRNAs exist in colon cancer tissues and patient blood, and are important in the pathogenesis of colon cancer (15). In the *in vitro* experiments performed in the present study, the expression of miRNA-766 in patients with colon cancer was increased, compared with that in the control group. The OS and DFS rates of patients with colon cancer and a high expression of miRNA-766 were higher than those of patients with colon cancer and a low expression of miRNA-766. Oh *et al* showed that miRNA-766 affected the distant metastatic process to a greater extent than cancer cell proliferation and primary tumor growth of human triple-negative breast cancer cells (16). Jia *et al* showed

that miR-766-5p suppressed the tumor growth of colorectal cancer (17). However, the present study used only one cell line, Caco2 cells, which is insufficient for this investigation.

miR-191, which locates in human 3p21.3, has been found to be overexpressed in numerous types of human tumor (18). For example, a high expression of miR-191 has been found in liver, stomach, large intestine, prostate, and breast cancer (19). It was found in liver cancer that miRNA-191 promotes epithelial-mesenchymal transition and exerts its tumor-promoting effect through suppressing the expression of TIM3; it may serve as a novel target in the treatment of liver cancer (15). It was confirmed in a study on gastric cancer that miRNA-191 promotes gastric cancer cell growth and inhibits cell apoptosis through its target gene NDST1 (15). In the present study, the overexpression of miRNA-766 reduced cell growth and cell migration, and promoted LDH activity, apoptotic rate and caspase-3/9 activity levels in Caco2 cells.

Colon cancer is one of the most common malignancies clinically. As indicated in numerous studies, tumorigenesis and development is linked with disruption to the dynamic balance between cell proliferation and apoptosis (19). Bcl-2 family proteins are important regulatory factors of cell

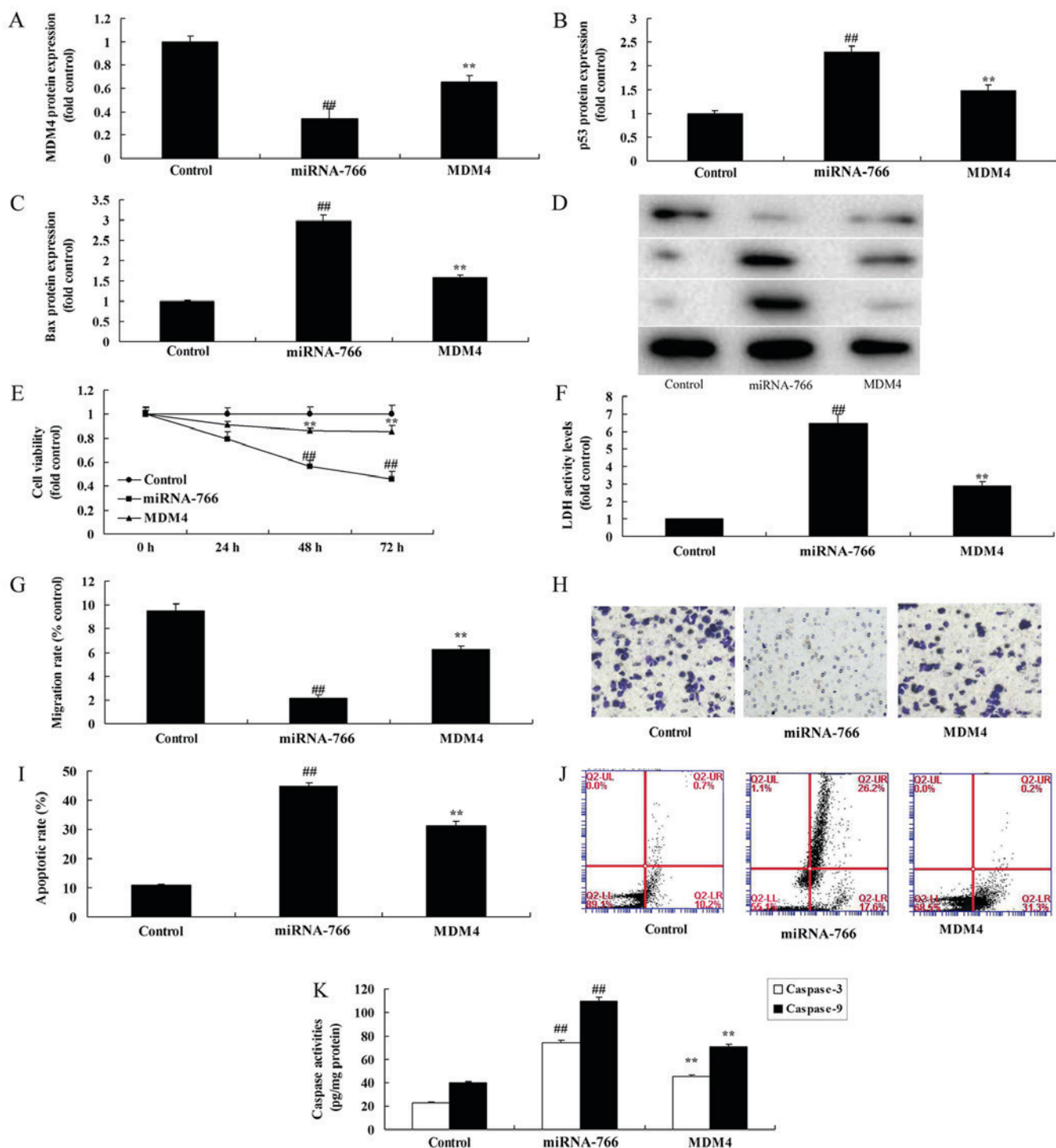


Figure 5. Promotion of MDM4 reduces the anticancer effect of miRNA-766 in colon cancer cells. Statistical analysis of protein expression levels of (A) MDM4, (B) Bax and (C) p53 from (D) MDM4, Bax and p53 protein bands. (E) Cell growth. (F) LDH activity. (G) Cell migration rate and (H) images. Magnification, $\times 100$. (I) Apoptotic rate quantified from (J) flow cytometry. (K) Caspase-3/9 activity levels. ## $P < 0.01$ vs. Control; ** $P < 0.01$ vs. miRNA-766. Control, negative control group; miRNA-766, overexpression of miRNA-766 group; MDM4, MDM4 and overexpression of miRNA-766 group. miR, microRNA; Bax, B-cell lymphoma 2-associated X protein.

of FL-MDM4 mRNA can be detected in colon cancer cells through RT-qPCR analysis, as reported previously (24). In the present study, the promotion of MDM4 reduced the anticancer effect of miRNA-766 in colon cancer cells. Wang *et al* also demonstrated that miRNA-766 induced p53 accumulation and G2/M arrest by directly targeting MDM4 in breast cancer (25).

In conclusion, the data obtained in the present study demonstrated that miRNA-766 reduced cell growth and cell migration,

and promoted LDH activity, apoptotic rate and caspase-3/9 activity levels through MDM4/p53 in Caco2 cells (Fig. 6). These findings provide a direct link between miR-766/MDM4 and human colon cancer survival rate and cell growth, which provides insight into the p53/Bax pathway. Additionally, these results support the hypothesis that genetic variants can interrupt miR-766-mediated gene regulation, and this type of regulatory gene may be important modifiers of human colon cancer risk.

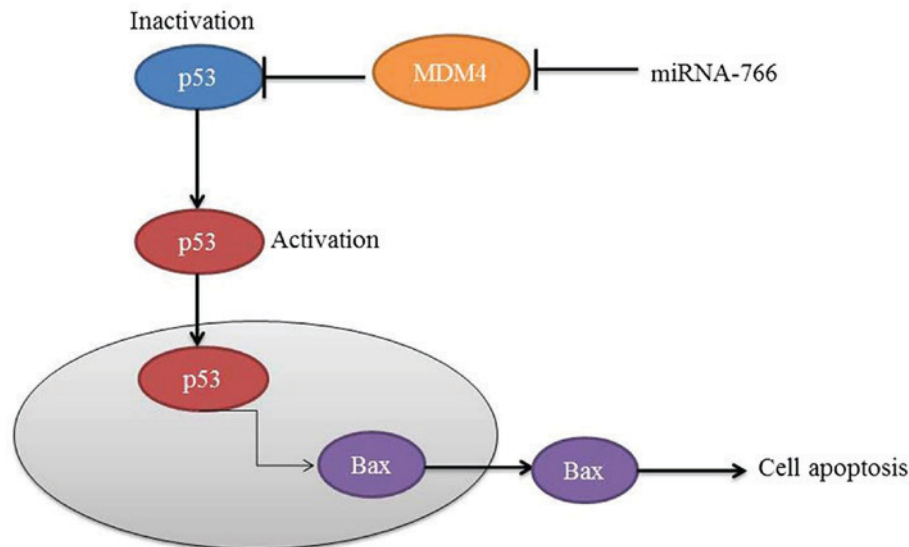


Figure 6. miRNA-766 induces cell apoptosis in human colon cancer through MDM4/p53. miRNA, microRNA; Bax, B-cell lymphoma 2-associated X protein.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

WC designed the experiments; GC, ZL, KL, GL and YL performed the experiments; WC and GC analysed the data; and WC wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Agent Will of the Second Affiliated Hospital, Shantou University Medical College.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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