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Study on the function and mechanism of long non-coding RNA DMTF1v4 in the occurrence of colon cancer

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Abstract. – OBJECTIVE: To investigate the expression of long non-coding RNA (lncRNA) DMTF1v4 in colon cancer, and the relationship between its expression and disease occurrence.

MATERIALS AND METHODS: Human colon cancer tissues and para-carcinoma tissues were harvested. The expression of lncRNA DMTF1v4 was measured by semi-quantitative PCR. The expression of DMTF1v4 in HT-29 colon cancer cells was downregulated using siRNA, and the effect of its downregulation on cell growth was determined by MTT assay and plate clone assay. The effect of DMTF1v4 downregulation on colon cancer cell migration was determined using a transwell assay and scratch wound assay. The effect of DMTF1v4 on colon cancer cell apoptosis was determined using Annexin V/PI double-staining. The changes in p-ERK, p-JNK, and p-p38 were measured by Western blot. HT-29 cells with downregulated DMTF1v4 expression were used to establish the subcutaneous heterotopic transplantation tumor model in nude mice to study the effect of DMTF1v4 on tumor growth in animals.

RESULTS: Compared with para-carcinoma tissue, lncRNA DMTF1v4 in colon cancer tissue was highly expressed ($p<0.001$). Downregulating lncRNA DMTF1v4 in HT-29 cells showed that lncRNA DMTF1v4 promotes cell proliferation and migration, and suppresses apoptosis ($p<0.05$). The effect of lncRNA DMTF1v4 on the ERK/MAPK signaling pathway was evaluated. The expression of p-ERK, p-JNK, and p-p38 was increased significantly compared with the control group ($p<0.01$). The effect of downregulating DMTF1v4 on tumor growth in animals showed that tumor growth in nude mice was decreased, and the expression of apoptosis-related proteins was increased ($p<0.01$).

CONCLUSIONS: The expression of lncRNA DMTF1v4 is elevated in colon cancer tissues; lncRNA DMTF1v4 promotes colon cancer cell proliferation and migration, and inhibits apoptosis by downregulating the expression of p-ERK, p-JNK, and p-p38, thus affecting the progression of colon cancer. This will provide a basis for the development of new clinical treatments for colon cancer.

Key Words:
Long non-coding RNA, DMTF1v4, Colon cancer, ERK/MAPK pathway.

Introduction

The occurrence and progression of colon cancer are closely related to changes in dietary patterns and improvements of life standard. In recent years, the incidence of colorectal cancer in China has increased significantly[1-3]. Furthermore, the colon cancer mortality ranks third among all malignant tumors. Then, colon cancer has become a major public health issue[4]. Presently, radical treatment of colon cancer primarily involves surgical excision, although the rate of mortality after surgery is still very high. Therefore, it is of great significance to determine the pathogenesis of colon cancer and search for new molecular targets for its treatment[5].

In the past, long non-coding RNA (lncRNA) was regarded as useless. With developments in molecular biology, the study of cell proliferation and apoptosis has progressed, and lncRNA was found to be closely related to these important cellular processes[6,7]. However, there are currently very few fundamental studies and clinical applications of lncRNA in colon cancer. Several lncRNA species associated with colon cancer have been found, but their roles and regulatory mechanisms remain unclear. LncRNA DMTF1v4 is highly expressed in a variety of human cancer cells, and it is closely related to the occurrence

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and progression of cancer[8,9]. The aim of the present work was to examine the relationship between the expression of lncRNA DMTF1v4 and the occurrence of colon cancer. We investigated the expression of lncRNA DMTF1v4 in colon cancer to determine a new pathogenic mechanism of colon cancer and to find new targets for its treatment.

Patients and Methods

Patients

Samples were collected from patients with colon cancer treated in Shenzhen Second People's Hospital from July 2016 to December 2016. The study was approved by the Ethics Committee of Shenzhen Second People's Hospital, First Affiliated Hospital of Shenzhen University. Signed written informed consents were obtained from all participants before the inclusion. Patients were diagnosed with colon cancer after pathological examination of colon cancer tissues. Patients with other wasting diseases were excluded, and they all signed the informed consent. Para-carcinoma tissue referred to tissue that was over 3 cm away from the cancer tissue in the same patient, and contained no cancer cells by pathological examination. A total of 38 patients underwent follow-up in our hospital after surgery. Harvested samples were stored in liquid nitrogen.

Equipment and Reagents

HT-29 cell line (Kunming Cell Bank of Chinese Academy of Sciences, Kunming, China), methyl thiazolyl tetrazolium (MTT) (Sigma-Aldrich, St. Louis, MO, USA), DMSO (Sigma-Aldrich, St. Louis, MO, USA), cell apoptosis kit (Beyotime Biological Technology Co., Ltd, Nanjing, China), transwell chamber (Millipore, Billerica, MA, USA) rabbit anti-human Bcl-2 antibody, rabbit anti-human Bax antibody, rabbit anti-human cleaved caspase 3 antibody, rabbit anti-human caspase 3 antibody, rabbit anti-human ERK antibody, rabbit anti-human p-ERK antibody, rabbit anti-human JNK antibody, rabbit anti-human p-JNK antibody, rabbit anti-human p38 antibody, rabbit anti-human p-p38 antibody, sheep anti-rabbit secondary antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-human β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA), Elite ESP-type flow cytometer (Coulter, Brea, CA, USA), fluorescent inverted microscope (Thermo Fisher, Waltham, MA, USA), cell culture bottles (Corning, Corning, NY, USA), Pipette (Eppendorf, Germany), gel electrophoresis imaging analysis system (Alpha Innotech, San Leandro, CA, USA) and qRT-PCR instrument (ABI, Vernon, CA, USA).

Detection of lncRNA DMTF1v4 Expression by Semi-Quantitative PCR

A TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from colon cancer tissue and para-carcinoma tissue, and cDNA was obtained by reverse transcription using a reverse transcription kit (Invitrogen, Carlsbad, CA, USA). GAPDH was taken as the internal reference, and the transcriptional levels of lncRNA DMTF1v4 in each tissue sample were measured. Primers were synthesized by Tiangen Biotech (Beijing, China), and the sequences are shown in Table I. The thermal conditions for PCR were as follows: the reaction temperature was 95°C for 5 min, 94°C for 1 min, 65°C for 30 s, 72°C for 45 s, for a total of 30 cycles, and extension at 72°C for 7 min.

siRNA-Mediated Down-Regulation of DMTF1v4

The DMTF1v4-siRNA and its corresponding control sequence were designed and synthesized by NimbleGen; HT-29 cells under healthy growth conditions were selected and transfected with the aforementioned siRNA. After 48 h of transfection, RNA was extracted and cDNA was obtained by reverse transcription. The expression of DMTF1v4 was measured by RT-PCR to determine whether transfection was successful. Successfully transfected cells were divided into the

Table I. PCR primers.

	Sequence
DMTF1v4	Forward primer: 5'- ACCCACAGACAACTGTGGACCC-3' Reverse primer: 5'- GCCGCCCCTATTGTTGCCCA-3'
GAPDH	Forward primer: 5'- GCACCGTCAAGGCTGAGAAC -3' Reverse primer: 5'- TGGTGAAGACGCCAGTGGA-3'

Table II. Tumor growth in nude mice after 4 weeks.

Test indexes	Control	siRNA-control	DMTF1v4-siRNA
Tumor volume (mm^3)	587. 8±36.6	532.7±32.5	282.8±25.6**
Tumor weight (mg)	483.3±22.8	492.9±21.7	260.3±18.5**

Note: Compared with the control group, the tumor volume and weight were significantly decreased in the DMTF1v4-siRNA group, $^{**}p<0.01$.

experimental group (DMTF1v4-siRNA), negative control group (control-siRNA), and blank control group (control).

MTT Assay and Plate Clone Assay

MTT assay and plate clone assay were conducted to determine the effect of downregulating DMTF1v4 on colon cancer cell growth. MTT assay: 5 mg/ml MTT was prepared, and 3×10^4/ml cells were seeded in 96-well plates. MTT was added after 48 h, and the culture medium was discarded 4 h after further incubation of cells. After adding dimethyl sulfoxide (DMSO), MTT and cells were shaken for mixing, and a microplate reader was used to measure the absorbance value at 490 nm. Plate clone assay: 5×10^5/ml cells were seeded in 6-well plates, and cultured until clone formation was significantly visible. Next, grid scotch tape was used for cell counting after washing, fixation, and staining[10].

Transwell Assay and Scratch Wound Healing Assay

The effect of downregulating DMTF1v4 on colon cancer cell migration was studied using a transwell assay and scratch wound-healing assay. Transwell assay: after cells were starved for 24 h, 5×10^5/ml cells were added to transwell chambers, and the number of cells crossing the chamber was calculated under the microscope after staining and fixation [11]. Scratch wound healing assay: the stable cell lines described above were seeded in 6-well plates, and cultured overnight using the same methods as described above. The wells were marked with 3-4 lines, and the scratched cells were discarded after two washes. Unscratched cells were added to serum-free culture medium for incubation for 16 h, followed by sampling and photographing.

Detection of Apoptosis by Flow Cytometry

A total of 5×10^5/ml cells were prepared as a single-cell suspension, seeded in culture plates, and incubated for 24 h. The cell suspensions were centrifuged and the supernatants were discarded. After adding Annexin V, the suspensions were incubated for 10 min at room temperature, followed by staining with PI. Cells were then analyzed by flow cytometry after incubation for 30 min at 4°C in the dark.

Establishment of a Subcutaneous Heterotopic Transplantation Tumor Model in Nude Mice

After silencing DMTF1v4 using lentivirus transfection, cells in the logarithmic growth phase were collected, and the cell density was adjusted to 5×10^7/ml. Under sterile conditions, cells were inoculated into nude mice by caudal vein injection, and the growth status of tumors was observed.

Effect of DMTF1v4 Down-Regulation on Tumor Growth

After successful establishment of the heterotopic transplantation tumor model in nude mice, the state of tumors was observed and size changes were recorded. After 4 weeks, tumor tissues were removed from liquid nitrogen, and cut using scissors. The supernatant was obtained after centrifugation and total protein was obtained. Protein samples were quantified, and equal amounts of protein were analyzed by Western blot. After development and exposure of protein bands, changes in the levels of the apoptosis-related proteins, Bax, Bcl-2, caspase 3, and cleaved caspase 3 were analyzed.

Statistical Analysis

Data are presented as mean ± standard deviation. SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used for the x^2-test and analysis; $p<0.05$ was considered as statistically significant.

Results

Expression of lncRNA DMTF1v4 in Colon Cancer Tissues

The levels of DMTF1v4 in 38 colon cancer tissue samples and 38 para-carcinoma tissue sam-

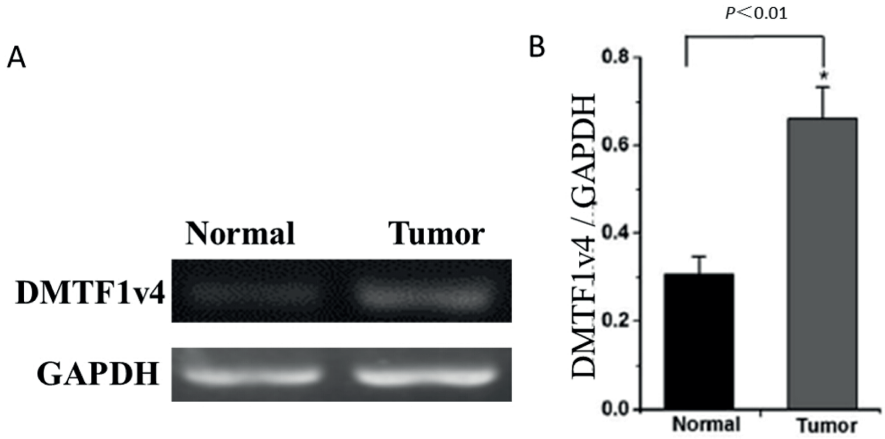

Figure 1. Detection of DMTF1v4 expression in colon cancer tissue by semi-quantitative PCR. ***A,*** Results of agarose gel electrophoresis; compared with the normal group, DMTF1v4 expression in colon cancer tissues was significantly increased. ***B,*** Statistical analysis; the difference in DMTF1v4 expression in colon cancer tissue and normal tissue was statistically significant, $^{**}p<0.01$.

ples were measured (Figure 1). Compared with para-carcinoma tissues, DMTF1v4 in colon cancer was highly expressed ($p<0.01$).

Establishment of the HT-29 Cell Line with Downregulated Expression of DMTF1v4

siRNA transfection was performed to downregulate the expression of DMTF1v4 in HT-29 colon cancer cells. RNA was then extracted. After reverse transcription, semi-quantitative PCR was used to measure the expression of DMTF1v4 (Figure 2). Compared with cells transfected with the siRNA-control, the expression of DMTF1v4 was decreased to 41.82 ± 3.25% ($p<0.01$) in cells transfected with DMTF1v4-siRNA. This suggested that the HT-29 cell line with downregulated expression of DMTF1v4 was successfully established.

Effect of Downregulated Expression of DMTF1v4 on Cell Growth

MTT assay was performed to determine the effect of DMTF1v4 on cell proliferation (Figure 3). Compared with the control group, the cell survival rate in the siRNA-control group was not significantly different. Compared with the control group, the cell survival rate in the DMTF1v4-siRNA group was significantly decreased. Therefore, after DMTF1v4 was downregulated, cell proliferation was significantly decreased ($p<0.01$). Furthermore, the plate clone assay was performed (Figure 4). Compared with the control group, cell proliferation in the siRNA-control group did not change significantly, while cell proliferation in the DMTF1v4-siRNA group was significantly decreased compared with the control group ($p<0.01$).

Effect of Downregulated Expression of DMTF1v4 on Cell Migration

Transwell assay was conducted to determine the effect of DMTF1v4 on cell migration (Figure 5). After DMTF1v4 was downregulated, cell migration ability was significantly decreased ($p<0.01$). In addition, the scratch wound healing assay was conducted (Figure 6). Compared with the control group, cell migration ability in the DMTF1v4-siRNA group was decreased significantly ($p<0.05$).

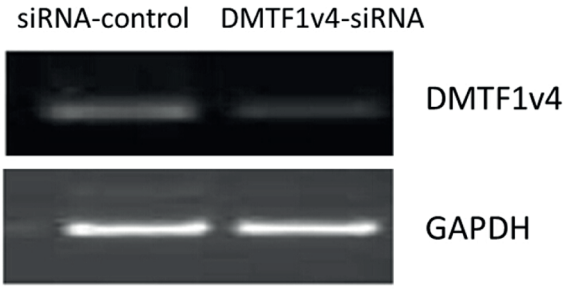

Figure 2. Detection of DMTF1v4 expression in HT-29 cells by semi-quantitative PCR. Agarose gel electrophoresis showed that compared with cells treated with siRNA-control, the expression of DMTF1v4 in cells treated with DMTF1v4-siRNA was significantly decreased ($p<0.01$).

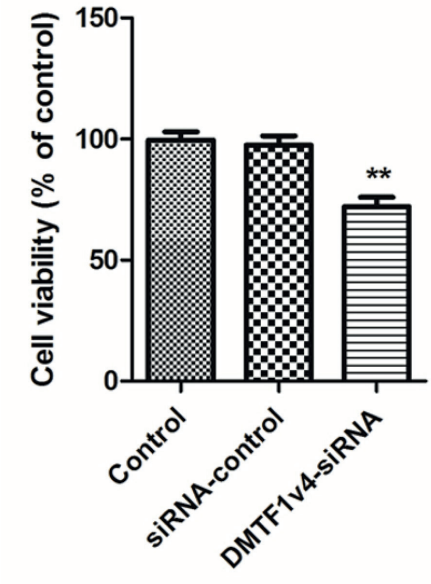

Figure 3. Determination of cell proliferation by MTT assay. Compared with the control group, the cell survival rate in the siRNA-control group was not significantly different ($p>0.05$). Compared with the control group, the cell survival rate in the DMTF1v4-siRNA group was decreased significantly, $^{**}p<0.01$.

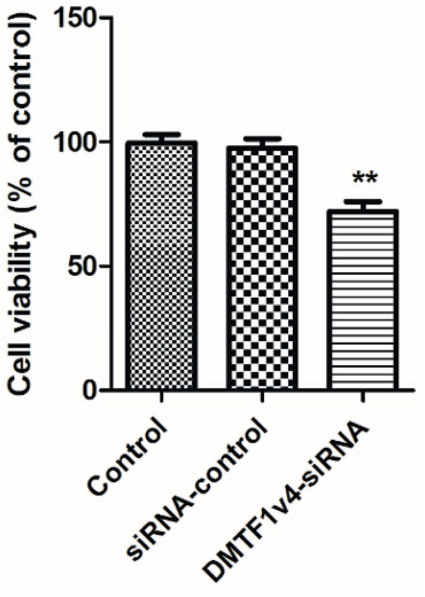

Figure 4. Detection of cell proliferation by the plate clone assay. Compared with the control group, the cell proliferation rate in the siRNA-control group was not significantly different ($p>0.05$). Compared with the control group, the cell proliferation rate in the DMTF1v4-siRNA group was significantly decreased, $^{**}p<0.01$.

Effect of Downregulated Expression of DMTF1v4 on Cell Apoptosis

To further explore the mechanism of DMTF1v4 on cell growth, flow cytometry was used to measure HT-29 cell apoptosis after transfection for 24 h (Figure 7). After DMTF1v4 was downregulated, cell apoptosis (15.4%) was increased significantly compared with the control group (3.5%) ($p<0.01$).

Expression of ERK/MAPK Signaling Pathway Proteins

To explore the mechanism of DMTF1v4 on cell apoptosis, the expression of related proteins of the ERK (extracellular signal-regulated kinase)/MAPK(mitogen-activated protein kinase) signaling pathway was measured (Figure 8). After DMTF1v4 expression was downregulated, the expression of p-ERK was significantly upregulated ($p<0.01$), indicating that ERK was activated. Furthermore, downregulation of DMTF1v4 increased the expression of p-JNK and p-p38 ($p<0.01$), indicating that DMTF1v4 has a regulatory effect on the ERK/MAPK signaling pathway.

Effect of downregulating DMTF1v4 on tumor growth in nude mice

The heterotopic transplantation tumor model in nude mice was established via caudal vein injection. After 4 weeks, the tumor volume and weight were measured. Compared with the control group, tumor volume and weight were significantly decreased ($p<0.01$), indicating that inhibiting DMTF1v4 expression can significantly inhibit tumor growth (Table II). Moreover, the expression of apoptosis-related proteins was measured by Western blot. The expression of the pro-apoptotic protein, Bax, was upregulated significantly, and the Bax/Bcl-2 ratio was increased significantly. Downregulation of DMTF1v4 resulted in upregulation of the expression of cleaved caspase 3 expression, and the ratio of cleaved caspase 3/caspase 3 increased significantly ($p<0.01$, Figure 9).

Discussion

There are various methods of treatment for colon cancer including chemotherapy, radiotherapy, and surgical resection. However, the therapeutic effect and prognosis are poor because of its rapid progression and high degree of deterioration, ultimately resulting in a high rate of mortality [12]. With rapid developments of techniques in molecular biology, the pathogenesis of colon cancer and the search for treatments have become areas of intense research[13]. Several in-depth studies

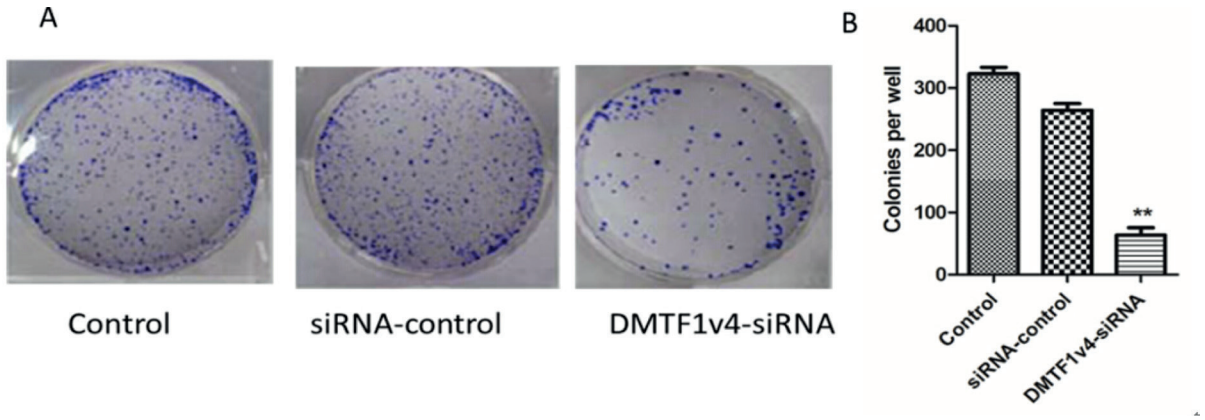

Figure 5. Determination of cell migration ability by transwell assay. ***A,*** Representative images of cells in transwell chambers; compared with the control group, cell migration ability in the DMTF1v4-siRNA group was decreased; ***B,*** Bar graph; compared with the control group, cell migration ability in the siRNA-control group was not significantly different ($p>0.05$); compared with the control group, cell migration ability in the DMTF1v4-siRNA group was significantly decreased ($p<0.01$).

on the pathogenesis of colon cancer aim to determine new pathogenic mechanisms of the disease and new molecular targets for clinical treatment. Studies have reported that lncRNA DMTF1v4 is highly expressed in a variety of human cancer cells, and is closely related to the occurrence and progression of cancer. However, there is no related literature on the relationship between the expression of lncRNA DMTF1v4 and the occurrence and progression of colon cancer[14]. In this study, colon cancer tissue and para-carcinoma tissue were compared, showing that the expression of lncRNA DMTF1v4 in colon cancer tissue was significantly higher compared with para-carcinoma tissue, consistently with the observation in liver cancer[15]. This indicates that lncRNA DMTF1v4 has a close correlation with the occurrence of cancer. Furthermore, downregulating lncRNA DMTF1v4 significantly inhibited cell proliferation and migration, and significantly increased cell apoptosis. At present, a large number of investigations have shown that lncRNA is closely

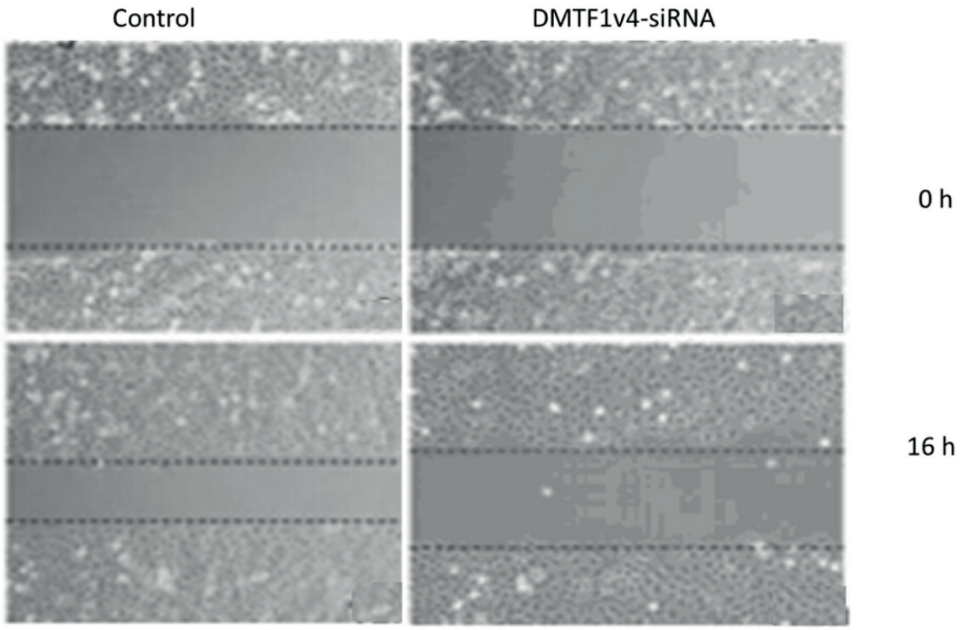

Figure 6. Determination of cell migration ability by scratch wound healing assay. Compared with the control group, cell migration ability in the DMTF1v4-siRNA group was significantly decreased ($p<0.05$).

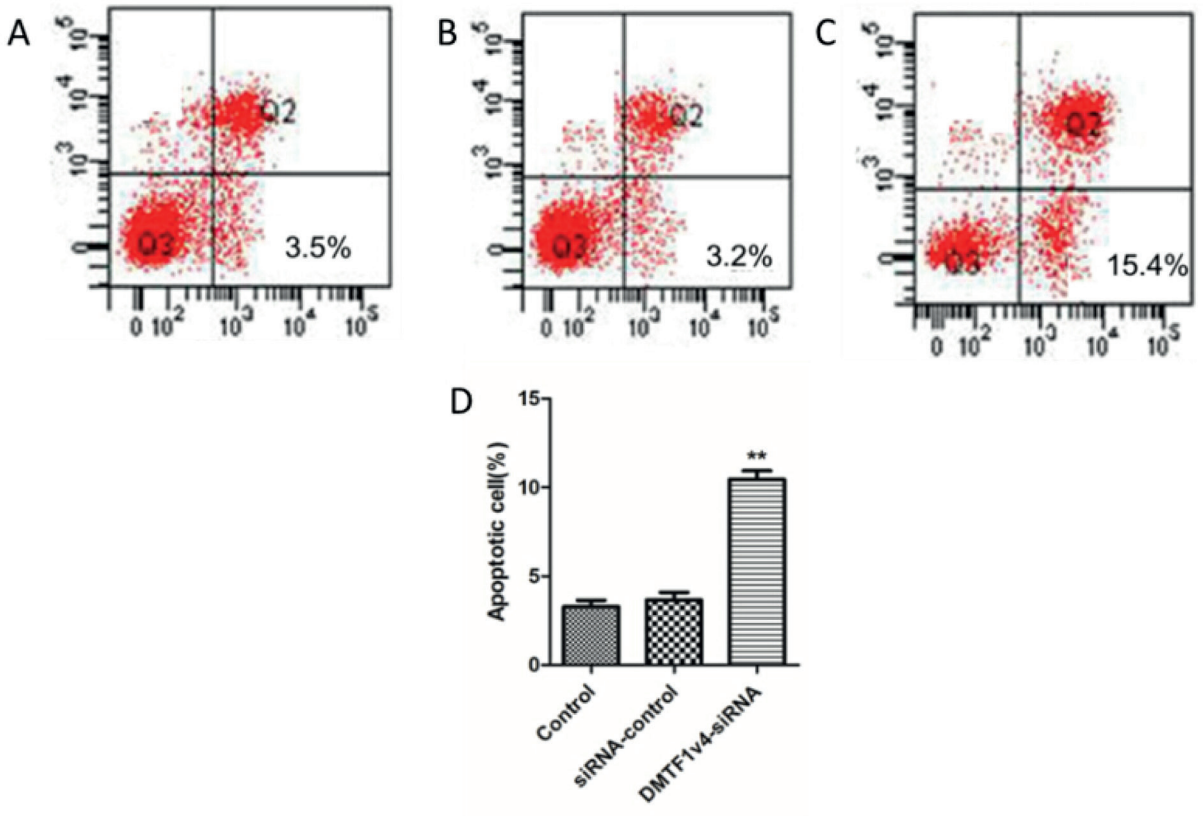

Figure 7. Detection of cell apoptosis by flow cytometry. ***A,*** Cell apoptosis in the control group, ***B,*** Cell apoptosis in the siRNA-control group, ***C,*** Cell apoptosis in the DMTF1v4-siRNA group, ***D,*** Statistical analysis; compared with the control group, cell apoptosis in the siRNA-control group was not significantly different ($p>0.05$); compared with the control group, cell apoptosis in the DMTF1v4-siRNA group was significantly increased.

related to human disease. LncRNA participates in the occurrence and progression of a variety of tumors, primarily by affecting cell proliferation, migration, and apoptosis, thus promoting the progression of cancer[16]. Gramantieri et al[17] found that monitoring the levels of lncRNA can predict the postoperative prognosis of liver cancer. Researches have shown that cancer cell proliferation and migration are closely related to the expression of each protein in the MAPK signaling pathway. Abnormal MAPK pathway regulation may lead to the deterioration and metastasis of a variety of tumors, and may severely affect other tissues and organs[18,19]. The MAPK pathway has a number of branches. ERK, a branch of the MAPK pathway, is often closely related to the activation of the MAPK pathway. p-ERK is produced by activating ERK. It regulates cell growth and differentiation, and acts to maintain cellular activity. The activation of ERK signaling pathways can also promote the activation of JNK and p38 to p-JNK and p-p38, thus inhibiting cell proliferation. However, some scholars[20-22] are opposite to this point of view. We investigated the role of the ERK/MAPK signaling pathway in colon cancer by measuring the levels of proteins of the ERK/MAPK signaling pathway in colon cancer, and examined the expression of pro-apoptotic proteins. The expression of ERK, p38, and JNK protein was analyzed by Western blot. When cell proliferation was inhibited, the expression of p-ERK, p-JNK, and p-p38 was significantly increased ($p<0.01$). When p-ERK expression was increased, the expression of the pro-apoptotic proteins, Bax, and cleaved caspase 3, was also increased significantly. The Bax/Bcl-2 ratio and cleaved caspase 3/caspase 3 ratio were increased significantly, indicating that activation of ERK in colon cancer cells can upregulate Bcl-2, thus activating caspase 3 to trigger apoptosis. The results also showed that lncRNA DMTF1v4 regulates the occurrence and progression of colon cancer through ERK/MAPK signaling pathways. To more intuitively verify that DMTF1v4 participates in the occurrence and progression of tumors through the regulation of apoptosis-related proteins, we established a heterotopic transplanta-

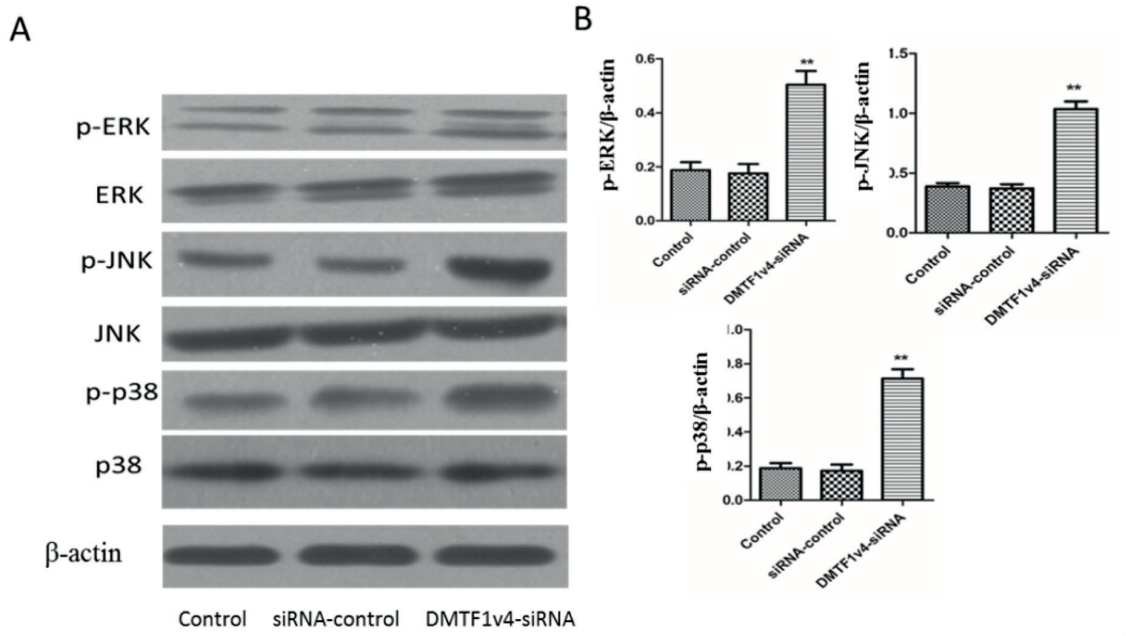

Figure 8. Detection of ERK/MAPK signaling pathway protein expression by Western blot. ***A,*** Representative Western blots. ***B,*** Statistical analysis; compared with the control group, the expression of related proteins in the siRNA-control group did not change significantly ($p>0.05$); compared with the control group, p-ERK, p-JNK, and p-p38 expression in the DMTF1v4-siRNA group was significantly increased ($p<0.01$).

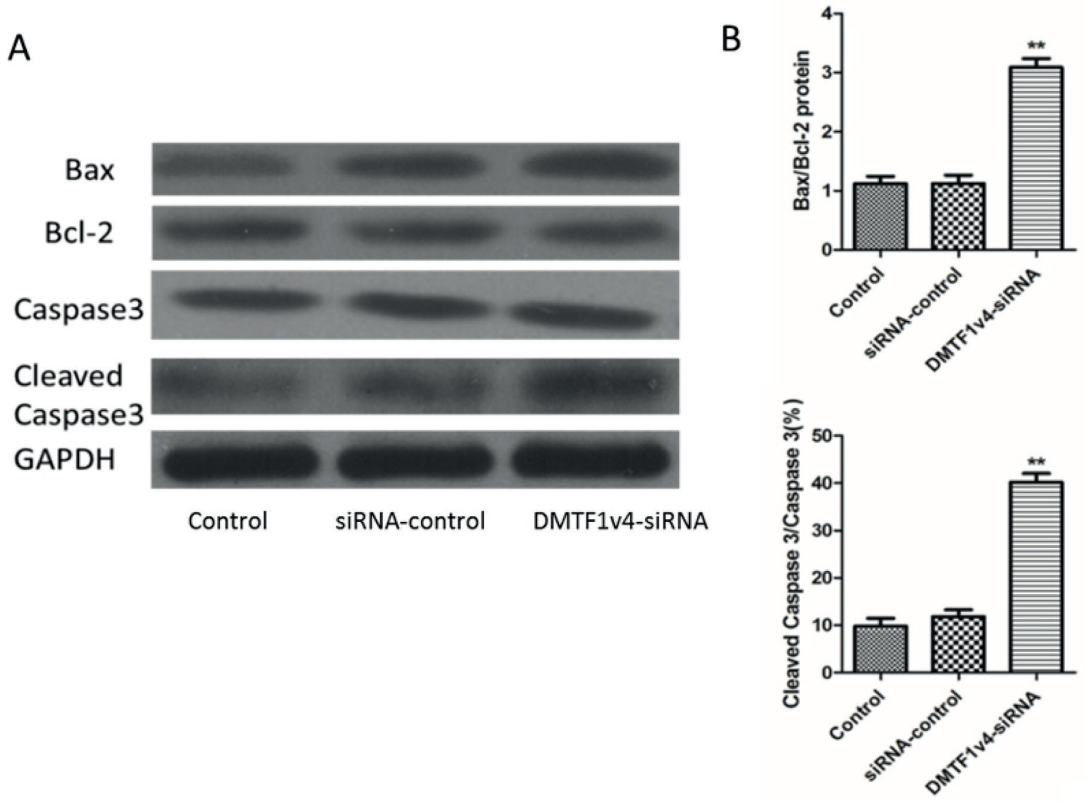

Figure 9. Measurement of apoptosis-related proteins by Western blot. ***A,*** Representative Western blots. ***B,*** Statistical analysis; compared with the control group, expression of the related proteins in the siRNA-control group was not significantly different ($p>0.05$); compared with the control group, the Bax/Bcl-2 ratio and cleaved caspase 3/caspase 3 ratio in the DMTF1v4-siRNA group were significantly increased ($p<0.01$).

tion tumor model in nude mice. When DMTF1v4 was downregulated, tumor weight and volume were decreased ($p<0.01$), and compared with the control group, the expression of apoptosis-related proteins in the model group was significantly increased ($p<0.01$).

Conclusions

We showed that increased expression of lncRNA DMTF1v4 in colon cancer was closely related to its occurrence. DMTF1v4 promoted colon cancer cell proliferation and migration, and inhibited cell apoptosis mainly through the downregulation of ERK/MAPK signaling pathways, thus affecting the progression of colon cancer. Our results may serve to promote the study of the molecular mechanisms of colon cancer and the identification of new targets for the clinical treatment of colon cancer.

Funding

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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