Knockdown of MON1B Exerts Anti-Tumor Effects in Colon Cancer In Vitro

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Source of support: Departmental sources

Background: Colon cancer is one of the most common cancers in the world. We performed the present study to determine the molecular mechanism of MON1B in colon cancer cells.

Material/Methods: Colon cancer tissues and adjacent normal tissues were collected from 34 colon cancer patients. MON1B-silenced LoVo colon cancer cells were constructed. RT-qPCR and Western blot analysis were used to detect mRNA and protein levels, respectively, of colon cancer tissues and cells. Cell counting kit-8 (CCK-8), wound healing, and Transwell assays were used to detect viability, migration, and invasion, respectively, of colon cancer cells.

Results: The mRNA and protein levels of MON1B were higher in colon cancer tissues and human colon cancer cell lines (HT-29, SW480, COLO205, LoVo). Cell proliferation, migration, and invasion abilities were all inhibited when MON1B was silenced in LoVo colon cancer cells. Both the mRNA and protein levels of tissue inhibitor of metalloproteinase (TIMP)-2 and iκB were increased, while that of matrix metalloproteinases (MMP)-2, MMP-9, metastasis-associated genes (MTA)-1, nuclear factor-kappa B (NF-κB), and chemokine receptor type (CXCR)-4 was decreased when MON1B was silenced.

Conclusions: MON1B interference exerted anti-tumor effect in colon cancer in vitro.

MeSH Keywords: Colonic Neoplasms • Neoplasm Metastasis • Receptor Activator of Nuclear Factor-kappa B

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/911002
**Background**

According to GLOBOCAN 2008, colon cancer is one of the most common malignant tumors in the world [1]. Nearly 1.2 million people worldwide were diagnosed as new cases of colon cancer each year in recent years, 60% of which were in developed countries [2]. Tens of thousands of people die from colon cancer each year [3]. Colon cancer accounts for 8% of all cancer deaths [4]. Colon cancer has become the fourth leading fatal malignancy in the world. Metastasis and recurrence are the main causes of poor prognosis in patients with colon cancer and are also the major problems faced by clinicians today [5]. The prognosis of patients with metastatic and recurrent colon cancer is significantly worse than that of earlier-stage patients [6]. Therefore, screening for colon cancer with metastatic potential and providing individualized treatment for patients with metastatic colon cancer are keys to improving the prognosis of patients with metastatic colon cancer. In the study of the development of colon cancer, researchers have been exploring metastasis-related mechanisms of colon cancer, identifying metastasis-associated genes and proteins, and providing molecular targets for treatment of metastatic colon cancer [7].

The occurrence, development, and metastasis of tumors are multi-stage and multi-step processes involving multiple genes and signaling pathways. The major current theories for distant metastasis of gastrointestinal tumors include direct invasion, lymphatic metastasis, and hematogenous metastasis [8]. Invasive tumor cells involve exocrine proteins such as metal matrix enzymes, which can decompose intercellular substances and increase the invasiveness of tumor cells [9]. In addition, the proteases secreted by tumor cells can also degrade the basement membrane, making tumor cells break through the basement membrane and spread directly to adjacent sites [10,11].

Nuclear factor-kappa B (NF-kB) is an important family of nuclear factors with various transcriptional regulation functions. It specifically binds to xB sequence, the enhancer of the x-linked gene of B-cell immunoglobulin [12]. The activation of NF-kB can regulate the expression of cytokines, adhesion molecules, chemokines, and immune receptor genes, affecting various biological functions such as cell differentiation, inflammatory response, immune response, apoptosis, and tumor growth [13]. Many studies have shown that NF-kB is closely related to human diseases, including colon cancer [14–16].

Yeast MON1 is an important docking factor for vacuolar fusion and is critical for apoptotic cell clearance [17]. The membrane recruitment of MON1B, a homolog of yeast MON1 protein in mammals, is essential for vesicular fusion of early endosomes [18]. Previous research has found that MON1B contains NF-kB binding sites and is correlated to the clinical stages and recurrence of prostate cancer [19]. Whether MON1B plays a role in colon cancer is still not clear.

In conclusion, we assessed the expression levels of Mon1b in colon cancer patients and investigated the molecular mechanism in colon cancer cells. Our findings may provide critical clues for how Mon1b regulates the progression of colon cancer and may lead to novel treatment strategies for colon cancer.

**Material and Methods**

**Patients and tissues**

We enrolled 34 colon cancer patients (18 men and 16 women) from Zhejiang Cancer Hospital (Hangzhou, China) from March 2015 to December 2016. The study was approved by the Ethics Committee of Zhejiang Cancer Hospital. All patients agreed to the experiments and provided signed informed consent. Colon cancer tissues and adjacent normal tissues 2 cm away from the cancer tissues were collected. The clinicopathological variables are presented in Table 1.

**Cell culture**

Human colon cancer cell lines (HT-29, SW480, COLO205, LoVo) and human colon mucosal epithelial NCM460 cells were

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**Table 1. The relationship of clinicopathological factors with MON1B levels.**

| Variables                  | MON1B levels | P value |
|----------------------------|--------------|---------|
|                            | Low (n=12)   | High (n=22) |     |
| Age (years)                | 67.5±4.5     | 66.3±3.1  | 0.365 |
| Gender                     |              |          |       |
| Male                       | 9            | 9        | 0.464 |
| Female                     | 6            | 10       |       |
| Pathological grade         |              |          |       |
| I–II                       | 10           | 8        | 0.009*|
| III–IV                     | 2            | 14       |       |
| Metastasis                 |              |          |       |
| No                         | 8            | 6        | 0.026*|
| Yes                        | 4            | 16       |       |
| Differentiation            |              |          |       |
| Well/moderate              | 9            | 7        | 0.016*|
| Poor                       | 3            | 15       |       |

* P<0.05, Chi-square test.
originally purchased from the American Type Culture Collection (ATCC) (Guangzhou, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gemini, USA) with fetal bovine serum (FBS, 10% (v/v)) (Gemini, USA), streptomycin (100 U/ml), and penicillin (100 μg/ml) (Invitrogen, USA) in an incubator (5% CO₂) at 37°C. The mRNA and protein levels of MON1B were detected by RT-qPCR and Western blot assays.

**siRNA interference**

The MON1B siRNA and the negative control siRNA were purchased from Thermo Fisher. LoVo cells were cultured to 70% confluence and then transfected with 30 pmol siRNA by Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer’s instructions.

**Cell viability assay**

Viabilities of colon cancer cells in different groups were measured using the Cell Counting Kit-8 (CCK-8; AbMole, USA). According to the protocols, cells (5×10⁴/well) were inoculated in 96-well plates. After being incubated for 12, 24, and 48 h, cells were stained with 10 μl CCK-8 staining reagent (final concentration at 10 nmol/L) for 1 h. The optical density (OD) values were measured using a 1500 microplate reader (Thermo, USA) at 450 nm.

**Wound healing assay**

Cell migration abilities of colon cancer cells in different groups were determined by wound healing assay. Briefly, cells (1×10⁵ cells/well) were inoculated into 12-well plates for 24 h and the confluent monolayer cells were scratched gently to form a cell-free zone and cultured at 37°C. After incubating for 12 and 24 h, the area of the cell-free zone was observed under DMi8 optical microscope (Leica, Germany).

**Transwell assay**

Invasion rates of colon cancer cells with MON1B interference were assessed and compared to Control and NC groups using 24-well Transwell chambers with 8-µm pore filters (Corning, USA) coated with Matrigel GFR (BD, USA). First, 5×10⁴ cells were inoculated in the upper chambers filled with DMEM culture media without FBS. The lower chambers were filled with DMEM culture media containing 10% FBS. After being incubated for 48 h, the lower membrane was fixed with 4% paraformaldehyde (PFA), stained with 0.1% crystal violet for 30 min at room temperature, and then rinsed with 10% acetic acid. The invaded cells were counted under a DMi8 optical microscope (Leica, Germany).

**Reverse transcription-quantitative PCR (RT-qPCR)**

The mRNA expression levels in tissues and cells were determined by RT-qPCR. The factors involve MON1B in colon cancer tissues and different cell lines, as well as cell invasion-related factors after MON1B was silenced in LoVo cells. Total RNA was extracted with TRizol Reagent (Invitrogen, USA), and 1 μg RNA was reversely transcribed to cDNA using EN-QuantiTect Reverse Transcription (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocols. PCR amplification was conducted using Fast SYBR Green Master Mix (Applied Biosystems, USA) and detected using an ABI 7900 Thermocycler (Applied Biosystems, USA): 25 s at 95°C for initial denaturation, 40 cycles setting (20 s at 95°C, 20 s at 57°C, 30 s at 72°C), and 10 min at 72°C for final extension. The primer sequences of MON1B, matrix metalloproteinases (MMP)-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-2, metastasis-associated antigen (MTA-1), NF-κB p65, IκB, Chemokine receptor type-4 (CXCR-4), and β-actin are displayed in Table 2.

**Western blot analysis**

Tissues or cells were lysed using RIPA Lysis Reagent (Boster, China) for 20 min, and then centrifuged at 10 000×g for 10 min on ice. Proteins in supernatant were harvested and quantified by BCA protein assay kit (Thermo Fisher, USA). Subsequently, 20 μg total proteins were subjected to each lane and separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Thermo Fisher, USA). After that, the membrane was blocked with 5% non-fat dry milk for 1 h at 37°C and probed with specific primary antibodies overnight at 4°C,

### Table 2. The primer sequences applied in the research.

| Name       | Type     | Sequence (5’-3’)   |
|------------|----------|-------------------|
| β-actin    | Forward  | GTGACATCGCCAAGAC  |
|            | Reverse  | GAAAGGTTGTAACGCAACT |
| MMP-2      | Forward  | CAGCGTGAAGTTTCCATT |
|            | Reverse  | GTGGCACAGAAAGGTGAGG |
| MMP-9      | Forward  | GAGACTCTACACCAGAGC |
|            | Reverse  | GAAAGTGAAGGGGAAAAGGC |
| TIMP-2     | Forward  | TCCAAGGGCCCTGAGAGAG |
|            | Reverse  | TGACTCTTACACCAGAGC |
| MTA-1      | Forward  | CTGCCGACCCACAGAAGA |
|            | Reverse  | TGTGCAGTCTGCTGCTG |
| NF-κB p65  | Forward  | CTACACAGGACACAGAGAC |  
|            | Reverse  | GGAAGGTTGTTGTGGTGC |
| IκB        | Forward  | TATCAGGAGAACCCAGAGA |
|            | Reverse  | GCCTTTGACTGTCCTCCG |
| CXCR-4     | Forward  | TGCATCGCCCTTCCCTT |
|            | Reverse  | TTCCCCTGCTCTGCTGCTG |

- **MON1B siRNA**
- **Cell Counting Kit-8 (CCK-8)**
- **Reverse Transcription (Qiagen, Valencia, CA, USA)**
- **RT-qPCR**
- **Western blot analysis"
including: rabbit anti-MON1B (Novus, NBP1-92131, 1: 2000), anti-MMP2 (Abcam, ab92536, 1: 1000), anti-MMP9 (Abcam, ab38898, 1: 1000), anti-TIMP-2 (Abcam, ab180630, 1: 1000), anti-MTA-1 (Abcam, ab, 1: 1000), anti-NF-κB (Abcam, ab16502, 1: 1000), anti-IκB (Abcam, ab32518, 1: 1000), anti-CXCR-4 (Abcam, ab181020, 1: 1000), and anti-β-actin (Abcam, ab8227, 1: 2000). Subsequently, the membranes were incubated with Goat Anti-Rabbit IgG H&L (HRP) secondary antibody (Abcam, ab6721, 1: 5000) for 1 h at 37°C. The immunoblots were visualized by enhanced chemiluminescence (ECL) detection reagents (Thermo Fisher, USA). Digital images of immunoreactive bands were analyzed by Bio-Rad ChemiDoc™ XRS+ System with Image Lab™ Software #1708265 (Bio-Rad, USA).

**Figure 1.** Higher expression levels of MON1B in colon cancer patients and cells were observed. (A) The mRNA expression levels of MON1B in 34 colon cancer patients were detected by RT-qPCR. (B) Representative Western blot results of MON1B are presented. (C) The survival of patients with higher and lower MON1B levels at 2 years were recorded. The mRNA (D) and protein (E) levels of MON1B in colon cancer cell lines (HT-29, SW480, COLO205, and LoVo) and human colon mucosal epithelial NCM460 cells were detected. * P<0.05 and ** P<0.01 vs. control.
Figure 2. MON1B interference inhibited cell proliferation, migration, and invasion abilities of colon cancer cells. The mRNA (A) and protein levels (B) of MON1B decreased remarkably in the si-MON1B group compared with Control and NC groups. (C) Cell proliferation abilities were inhibited in a time-dependent (12, 24, and 48 h) manner in the si-MON1B group compared with Control and NC groups. (D, E) Cell migration abilities of colon cancer cells were inhibited in a time-dependent (12 and 24 h) manner in the si-MON1B group as detected by wound-healing assay. (D, F) Cell invasion abilities of colon cancer cells were significantly also inhibited. * $P<$0.05 and ** $P<$0.01 vs. Control group; * $P<$0.05 and ** $P<$0.01 vs. NC group.
Statistical analysis

SPSS 22.0 statistical software (SPSS, USA) was used to conduct statistical analysis; the values were derived from at least 3 independent repeated experiments and are presented as mean ± standard deviation. P<0.05 was regarded as a significant difference as calculated by Dunnett’s post hoc test.

Results

The higher expression levels of MON1B in colon cancer patients and cells were observed

To determine the expression levels of MON1B in non-small cell colon cancer patients, we performed RT-qPCR and Western blot analysis to detect MON1B levels in 34 colon cancer patients. The colon cancer tissues and adjacent normal tissues were used to compare the mRNA and protein levels. The results showed that the mRNA and protein levels of MON1B were elevated dramatically in most of colon cancer tissues compared with adjacent tissues (P<0.05, Figure 1A, 1B). All the MON1B mRNA expression diagrams and representative protein images are shown. The MON1B levels were correlated with tumor differentiation, TNM stages, and metastasis degrees (Table 1). The survival analyses of patients with higher and lower MON1B levels at 2 years were compared to each other, which showed that patients with lower MON1B levels lived longer than patients with higher levels (Figure 1C). Besides tissues, MON1B levels were measured in 4 colon cancer cell lines (HT-29, SW480, COLO205, LoVo) and human colon mucosal epithelial NCM460 cells. The results showed that the mRNA and protein levels of MON1B increased notably in all detected colon cancer cells, especially LoVo cells, compared with normal colon cells (P<0.05, Figure 1D, 1E). Hence, we chose LoVo cells to transform siMON1B to knock it down and observe related variations.

Knockdown of MON1B inhibited cell proliferation of colon cancer cells

The interfering efficiency of si-MON1B on LoVo colon cancer cells was evaluated by RT-qPCR and Western blot assays, which indicated that mRNA and protein levels of MON1B decreased significantly in the si-MON1B group compared with Control and NC groups (P<0.05, Figure 2A, 2B). Then, CCK8 assay was performed to measure the effect of MON1B on cell viability of
colon cancer cells, which was inhibited time-dependently (12, 24, and 48 h) in the si-MON1B group, with significant differences at 24 and 48 h ($P<0.05$, Figure 2C).

**Knockdown of MON1B inhibited cell migration and invasion abilities of colon cancer cells via inhibiting MMP-2, MMP-9, and MTA-1, as well as promoting TIMP-2**

To determine the effect of MON1B on cell migration and invasion abilities of colon cancer cells, we tested them using wound healing and Transwell assays, respectively. The results showed that the wound healing rates were time-dependently (12 and 24 h) and significantly inhibited when MON1B was knocked down in LoVo colon cancer cells compared with the Control and NC groups ($P<0.05$, Figure 2D, 2E) and the invasion abilities were also inhibited significantly after MON1B was knocked down ($P<0.05$, Figure 2D, 2F). The expression levels of MMP-2, MMP-9, TIMP-2, and MTA-1 were analyzed by RT-qPCR and Western blot analysis, respectively. The results showed that the mRNA and protein levels of MMP-2, MMP-9, and MTA-1 were significantly decreased in the si-MON1B group ($P<0.05$, Figure 3A, 3B, 3D, 3E), while TIMP-2 was significantly increased in the si-MON1B group ($P<0.05$, Figure 3C, 3E).

The knockdown of MON1B inhibited the NF-κB pathway

To illuminate the molecular mechanism of MON1B in colon cancer cells, we studied the relationship of MON1B with the NF-κB pathway. The observations showed that mRNA and protein levels of NF-κB p65 and CXCR-4 were remarkably decreased in the si-MON1B group, while that of IκB was significantly increased, compared with Control and NC groups ($P<0.05$, Figure 4A–4D).

**Discussion**

Because the main cause of colon cancer recurrence is tumor metastasis, it would be helpful to reveal novel genes regulating tumor metastasis for colon cancer treatment [20,21]. In the present study, we collected colon cancer tissues and adjacent normal tissues from 34 colon cancer patients and found that the mRNA and protein levels of NF-κB p65 and CXCR-4 were remarkably decreased in the si-MON1B group, while that of IκB was significantly increased, compared with Control and NC groups ($P<0.05$, Figure 4A–4D).

Figure 4. MON1B interference inhibited cell proliferation and migration of colon cancer cells via inhibiting the NF-κB pathway. (A–C) The mRNA levels of NF-κB p65, IκB, and CXCR-4 were measured by RT-qPCR. (D) The protein levels of NF-κB p65, IκB, and CXCR-4 were measured by Western blot. * $P<0.05$ and ** $P<0.01$ vs. Control group; * $P<0.05$ and ** $P<0.01$ vs. NC group.

**Journal Information**

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Jiang L. et al.: The role of MON1B on colon cancer cells © Med Sci Monit, 2018; 24: 7710-7718

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functioning in colon cancer, we did further research on colon cancer cells in vitro. The mRNA and protein levels of MON1B were also found to be significantly higher in the 4 common colon cancer cells: HT-29, SW480, COLO205, and LoVo cells. Among these, levels of MON1B in LoVo cells were the highest, so we chose LoVo cells for use in conduct MON1B interference experiments.

To further illustrate the molecular mechanism of MON1B interference in inhibiting cell proliferation, metastasis, and invasion abilities, we evaluated variations of metastasis-related factors, such as MMPs. MMPs are a family of zinc-dependent endopeptidases, degrading extracellular matrix and basement membrane [22]. MMP-2 and MMP-9 are the most important factors degrading type IV collagen, which is the main component of basement membrane, and is convenient to use in studies on for cell invasion and tumor metastasis [9]. The balance of MMPs and their inhibitor TIMP-2 co-regulate the progression of tumor metastasis [22]. MTA-1 is an important family of metastasis-associated genes. MTA-1 has been reported to be associated with TNM stages and differentiation degrees of colon cancer [23]. In our study, MON1B interference significantly inhibited the expression of MMP-2, MMP-9, and MTA-1, and promoted the expression of TIMP-2 in colon cancer cells. Our results indicate that the function of MON1B in colon cancer cells is closely related to the regulation of MMP-2, MMP-9, TIMP-2, and MTA-1 expressions.

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