

Original Article

MTAP reduces the development of colorectal cancer through mediating the NF- κ B pathway

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Abstract: Background: Colorectal cancer (CRC) is one of the gastrointestinal tumors. MTAP gene has a close relationship with the tumor and the NF- κ B pathway has been reported to be related with multiple diseases. This study was mainly focused on the role of MTAP and NF- κ B pathway in the development of colorectal cancer. Methods: Thirty pairs of colorectal tumor and tumor-adjacent tissue specimens were collected from our hospital and expression of MTAP was detected. RT-PCR was applied to test the mRNA expression of MTAP in five CRC cell lines of normal colonic cell line NCM460 (SW480, SW620, HCT116, HT29, and LoVo) and tumor tissues to examine metastasis. Relative expression of the NF- κ B pathway in both high and low MTAP expression LoVo cell lines was analyzed through Western blot. Western blot and immunofluorescence were used to detect inhibition of LoVo cell growth through suppression of the NF- κ B pathway by MTAP. Finally, flow cytometry and immunofluorescence tests were applied to determine cell apoptosis by suppressing the NF- κ B pathway through MTAP. Results: These data show that the expression of MTAP is lower in CRC cell lines when compared with the normal colonic cell line ($P < 0.05$). In addition, the metastasis CRC tissues also showed lower expression of MTAP than the non-metastasis CRC tissues. Ectopic expression of MTAP and RNA interference significantly reduced cell proliferation through the inhibition of NIK and enhanced cell apoptosis of CRC cells. Therefore, MTAP plays an important role in development of CRC by targeting NIK. Conclusion: Downregulation of MTAP leads to activation of the NF- κ B pathway and the malignant phenotype of CRC.

Keywords: MTAP, NF- κ B, colorectal cancer

Introduction

The generation and development of colorectal cancer (CRC) is a complicated process. In the Western world, colorectal cancer is the No. 2 killer in cancer-related diseases. Recently, advances in the treatment of colorectal cancer have successfully saved some patients with early-stage colorectal cancer. However, the mortality rate is still high for advanced patients. Therefore, there is an urgent need to understand the mechanism behind the development of CRC [1-3]. Methylthioadenosine phosphorylase (MTAP) plays a key role in the salvage of both adenine and methionine which transfers the methylthioadenosine (MTA) into the adenine and methylthioribose-1-phosphate into methionine (MTR-1-P). The existence and activity of MTAP protein have been discovered in both Caucasian and Asian and is called the 'Butler' enzyme. However, it is found that the

loss of MTAP exists in many different malignant tumors including leukaemia, lymphadenoma, mesothelioma, biliary carcinoma, glioblastoma, osteosarcoma, neuroendocrine tumor, lung cancer, breast cancer, pancreatic cancer and squamous-cell carcinoma. According to the tumor type and the method to evaluate the MTAP state, the loss rate ranges from 14% to 100%. Furthermore, the MTAP gene can be deactivated though the gene loss and promoter methylation. It was initially believed that the loss of MTAP in human tumor cells is only the result of closing to the CDKN2A/ARF tumor inhibition gene. However, recent studies have shown that MTAP has tumor inhibition function which is independent of CDKN2A/ARF loss [4-6]. Studies have also suggested that the loss of MTAP in tumor cells can block tumor cell differentiation through inducing G1 or G2/M cycle cell arrest and disturbing the mitosis, and ultimately affect tumor cell growth [7-9]. These results

reveal that the expression of MTAP is closely related to the tumor cell cycle, cell proliferation, and apoptosis. Moreover, expression of MTAP is related to the tumor cell metastasis and has a good value for prognosis [10, 11]. To date, very few studies have focused on the role and mechanism of MTAP in colorectal cancer.

The NF- κ B pathway is widely present in the cytoplasm and nucleus of mammalian cells. The pathway is highly conserved and involved in the regulation of inflammation signals. The NF- κ B pathway can be stimulated by different stimulators such as cytokine, neurotransmitter, hormone, and physical stimulation, activating a series of cascade amplification reactions [12]. The MAPK family includes eight subfamilies, including p50, p65, etc. Different NF- κ B pathways are activated by different signals and these pathways can cooperate with each other and lead to the synergistic effect or the inhibition effect. The most important and common pathways of NF- κ B are p50 and p65 [13]. A study has shown that the p50 pathway is mainly involved in the cell apoptosis and p65 pathway can affect the cell apoptosis by regulating the activation of caspase [14]. In addition, caspase can also activate the protein kinase in NF- κ B pathway and then induce p65 phosphorylation, leading to expression of Fas and p53 to induce cell apoptosis [15]. Nevertheless, expression of MTAP and its mechanism in the development of colorectal cancer needs elucidation.

In this study, the expression of MTAP in 30 selected colorectal cancer patients between July 2014 and February 2017 were examined. The regulatory role of MTAP on NF- κ B pathway was further investigated in colorectal cancer cell models.

Patients and methods

Patients

This study included 30 selected colorectal cancer patients with pathologic diagnosis from July 2014 to February 2017 in our hospital. The tumor tissues and adjacent non-cancerous tissues were collected. This study was approved by the Research Ethics Committee at the Jilin University. All patients/relative signed the informed consent to approve the use of tissue samples in this study.

Cell and culture

The cell lines used in this study including one normal colonic cell line NCM460 and five CRC cell lines (SW480, LoVo, HCT116, HT29, SW-620). All cell lines were purchased from Bena Culture Collection (Beijing, China). Cells were maintained in RPMI 1640 medium (Sigma, Gibco, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 100 u/ml penicillin, 100 μ g/ml streptomycin at 37°C in an incubator with 5% CO₂.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from tissue samples or cells by Trizol reagent (Invitrogen, Shanghai, China). The optical density (OD) of total RNA was measured using a spectrometer at the wavelength of 260 and 280. The expected ratio of OD/260/280 was between 1.8 and 2.0. The total RNA was reverse transcribed into cDNA using SuperScript II (Invitrogen). Real time PCR was performed using an America Superarray Corp.'s Master Mix premix system. The PCR reaction mixture (25 μ l) includes 12.5 μ l of SYBR Green Master Mix (TaKaRa, Qingdao, China), 10.5 μ l of H₂O, 1.0 μ l of template cDNA, 1.0 μ l of 10 μ M PCR primers. The reaction program was as follows: pre-denaturing, 95°C, 10 minutes; denaturing, 95°C, 30 seconds; annealing 55°C, 30 seconds; extension, 72°C, 30 seconds; 40 cycles. The following primers were synthesized by Genscript Biotech. (Nanjing, China): MTAP-forward: 5'-TACGGUCAUGUCCAAAGUTAT-3', reverse: 5'-ATUGUUGGACAUGACCGGAAC-3'; GAPDH-forward: 5'-GCCCTGAGGGCCCGAACTGTTACT-3'; and reverse 5'-CAGACGCACGGCTTTGACCTTCTT-3'. The experiment was repeated three times. Data was analyzed using 2^{- $\Delta\Delta$ Ct} method. GAPDH was used as the internal reference.

Stable transfection to LoVo cells with high and low expression of MTAP

The MTAP specific shRNA (si-MTAP) and negative control are purchased from Shanghai Gene Corp. LoVo cells were seeded at logarithmic phase into 6 well plates (10⁵ per well) and 50 nM si-MTAP or negative control were used to transfect the cells by HiPerFect transfection agent (German QIAGEN Corp.) for 48 hours. Overexpression and low expression MTAP plas-

mids and MTAP sequence and their corresponding negative controls were purchased from Guangzhou Ribo Biotech.

Western blot

Protein expression was determined by Western blot. Briefly, total cell protein was extracted and quantified using Bradford protein assay kit (Beyotime Biotech., Shanghai, China). Equal aliquotes (40 ug) of protein were then separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% skim milk for 30 minutes and incubated with rabbit anti-MTAP (1:1000 dilution, America CST Corp.) and anti-GAPDH (1:1000 dilution, America CST Corp.) The immunoreactivity was detected using the ECL detection system (Shanghai Gene Corp., Shanghai, China). Relative expression of proteins was quantified using Image J software (National Institutes of Health, Bethesda, USA). GAPDH was used as the internal reference.

Immunofluorescence

The LoVo cells were cultured on 6 well slides and then fixed by 4% paraformaldehyde for 10 minutes at -20°C. Then the slides were washed three times with PBS and incubated with NIK polyclonal antibody for 2 hours. After that they were washed with PBS three times, 5 minutes each and incubated with TRITC-antimouse IgG at room temperature for 1 hour. Washing the slides again with PBS was done next for three times and incubated with Hoechst 33258 for 5 minutes. Finally slides were washed and observe it under the fluorescence microscope (German Zeiss Crop.).

Hoechst 33258 staining

LoVo cells were cultured in the 6 well plates and then transfected with MTAP simulant, inhibitor and negative control for 48 hours. After that, washing the cells with PBS and staining with Hoechst 33258 (Beijing Solarbio Crop.) for 5 minutes and finally washed with PBS three times.

Quantification of cell apoptosis

The Annexin V- FITC kit (America Thermofisher Crop.) and flow cytometry are applied to quan-

tify the cell apoptosis. The LoVo cells were transfected with miR-518a-3p stimulant (50 nM) and negative control (50 nM) for 48 hours and then put into a 5 ml tube. After that the cells were washed with cold PBS and resuspended at the concentration of 10⁶/ml. Annexin V-FITC (5 ul) and propidium iodide were mixed with the cell and incubated at room temperature for 15 minutes. Incubation was done for another hour and flow cytometry was used to test the sample.

Statistical analysis

All results are shown as mean ± SD. The difference between two groups were analyzed by student's t-test. The difference among groups were compared by one-way ANOVA followed by post-hoc Tukey HSD test. The data are analyzed by SPSS 13.0 software and processed by GraphPad Prism 5.0 software. The significance level was set by *p* value <0.05.

Results

MTAP expression is down-regulated in colorectal tumor and cells

First, expression of MTAP in five CRC cell lines and normal colonic cell line NCM460 was determined. Compared with NCM460 cells, the expression of MTAP in CRC cell lines was significantly downregulated (*P*<0.05) (**Figure 1A**). Among five CRC cell lines, LoVo cells showed the lowest level of MTAP, the HCT116 cells showed the highest expression of MTAP (**Figure 1A**). In addition, the MTAP expression levels were determined in CRC tissues and non-metastasis normal tissues. It was noticeable that the level of MTAP in the CRC tissues was significantly lower than the normal tissues (*P*<0.01, **Figure 1B**).

MTAP negatively regulates the NF-κB pathway

Introduction of MTAP recombinant protein into the LoVo cells leads to downregulation of NIK in its protein level which is related to the down-regulation of the phosphorylation of IκBα and NF-κB activity (**Figure 2A** and **2B**). Compared with that, inhibition of MTAP causes the NIK protein accumulation in LoVo cells (**Figure 2C** and **2D**). These results exhibit that the level of MTAP is negatively related to the NF-κB activity

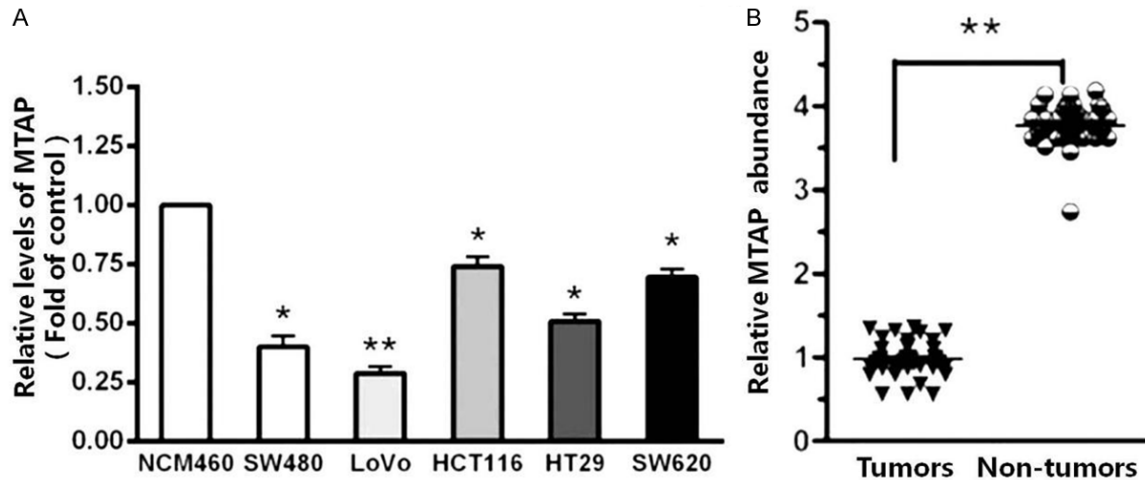


Figure 1. Relationship between expression of MTAP in colorectal tumor and clinical situation. A. Expression of MTAP in CRC cell lines and normal colonic cell line NCM460; B. Expression of MTAP in the tumor tissues and non-tumor tissues. *means $P < 0.05$, **means $P < 0.01$.

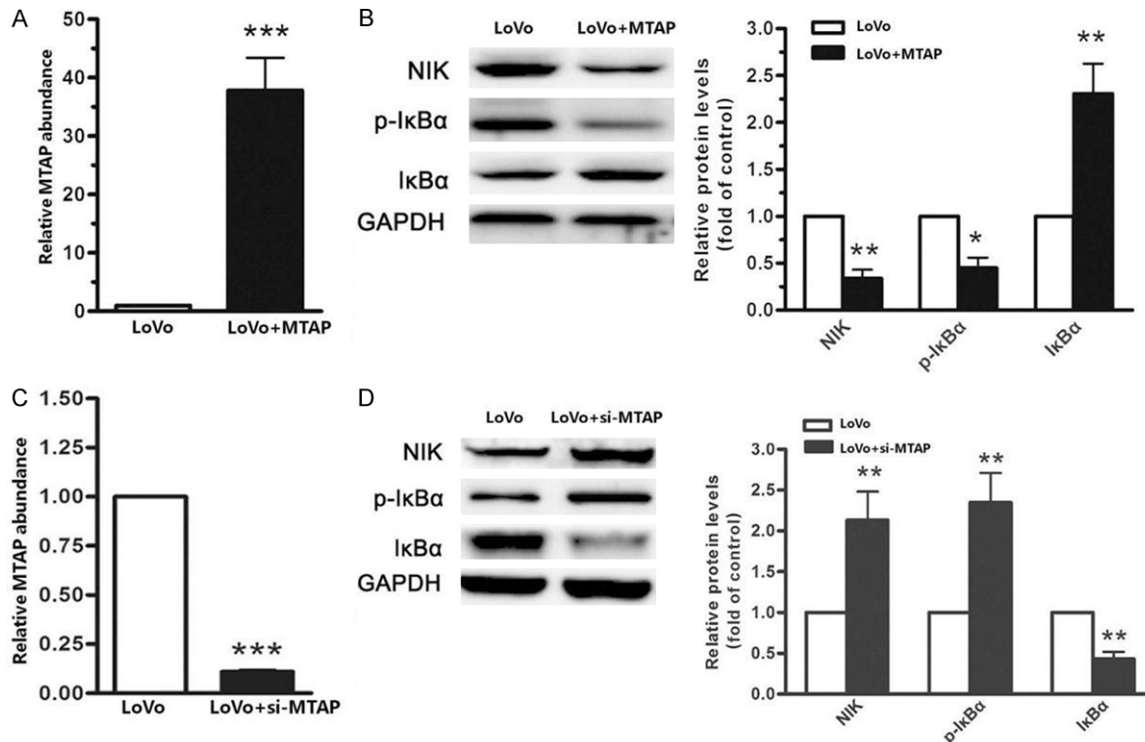


Figure 2. MTAP negatively regulates the NF- κ B pathway. A. High expression of recombinant MTAP protein in LoVo cell line. B. Western blot determines the expression of NF- κ B pathway related proteins and the right figure shows the statistical analysis results; C. Expression of MTAP after si-RNA interference; D. Expression of NF- κ B pathway related proteins after si-MTAP interference and the right figure shows the statistic results. *means $P < 0.05$, **means $P < 0.01$, ***means $P < 0.001$.

and the MTAP may inhibit the non-classical NF- κ B pathway and the activity of receptor promotor. Furthermore, MTAP may able to regulate expression of NIK and play a key role in the negative regulation of NF- κ B pathway.

MTAP inhibits LoVo cell growth by suppressing NF- κ B pathway

Studies have determined that the abnormal accumulation of NIK in cells can lead to the

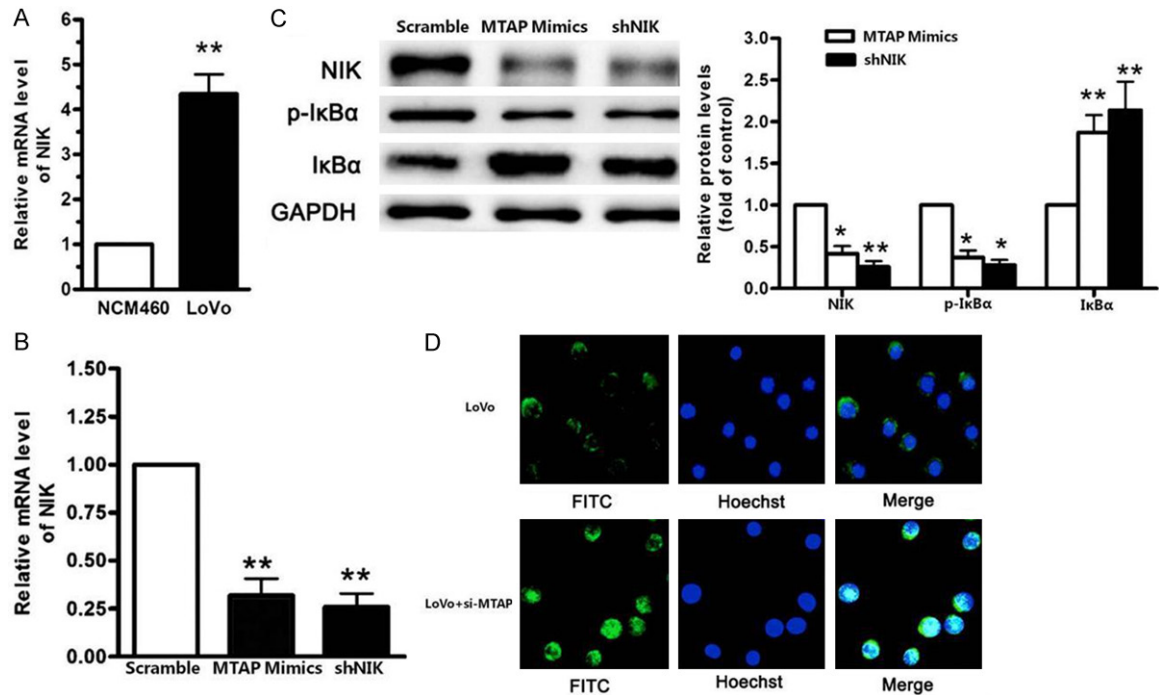


Figure 3. MTAP inhibits LoVo cell growth by suppressing the NF-κB pathway. A. Expression of NIK in NCM460 and LoVo cell lines. B. Expression of NIK in cell lines transfected with MTAP mimics and shNIK; C. Expression of NF-κB pathway related proteins and the statistical analysis results; D. Immunofluorescence test to locate the expression of NF-κB pathway. *means $P < 0.05$, **means $P < 0.01$.

activation of NF-κB pathway, however, the mechanism behind the over-produce of NIK is still unknown. The RT-PCR result shows that compared with NCM460 cells, the abnormal accumulation of NIK in LoVo cells can cause the activation of NF-κB pathway (Figure 4A). In order to understand the functions of NIK and MTAP, the NCM460 cell line was modified to stably express MTAP and NIK specific shRNA (shNIK) by using reverse transcription virus vectors. The RT-PCR and Western blot results exhibit that expression of MTAP or the transfection of shNIK reduce the mRNA and protein levels of NIK (Figure 3B and 3C). In addition, the RelA increase in the nucleus enhanced the activities of both classical and non-classical NF-κB pathways (Figure 3D). Re-expression of NIK led to reactivation of the NF-κB pathway that inhibited by MTAP which means there is a relationship between the levels of MTAP and NIK.

MTAP promotes cell apoptosis by inhibiting NF-κB pathway

The MTAP-regulated NF-κB pathway can affect cell apoptosis and many studies have shown

that activation of the NF-κB pathway can suppress cell apoptosis in CRC cells. Although inhibition of NIK by MTAP or shNIK led to downregulation of Bcl-xL, XIAP and FLIP genes which are involved in the cell apoptosis (Figure 4A), MTAP could suppress the activity of NF-κB pathway and cause apoptosis. In order to evaluate the biological function of MTAP as apoptosis signal, the MTAP stimulator was transfected into LoVo cells where it promoted LoVo cell apoptosis (Figure 4B). Additionally, overexpression of MTAP causes activation of caspase 3 (Figure 4C). In general, these results exhibit that MTAP causes the cell apoptosis through the inhibition of NIK in colonic cell lines. In order to prove the role of MTAP in the tumor cell survival, we test whether MTAP mimics transfection can kill the tumor cells and quantify the apoptotic cell numbers by Hoechst staining. The results show that the expression of MTAP do promote the tumor cell apoptosis (Figure 4D). In addition, since the shRNA which suppresses the NIK expression also has a strong killing effect, thus NIK and NF-κB may play key roles in the survival of CRC cells. In short, from this study, MTAP shows tumor inhibition in CRC cell lines and regulation

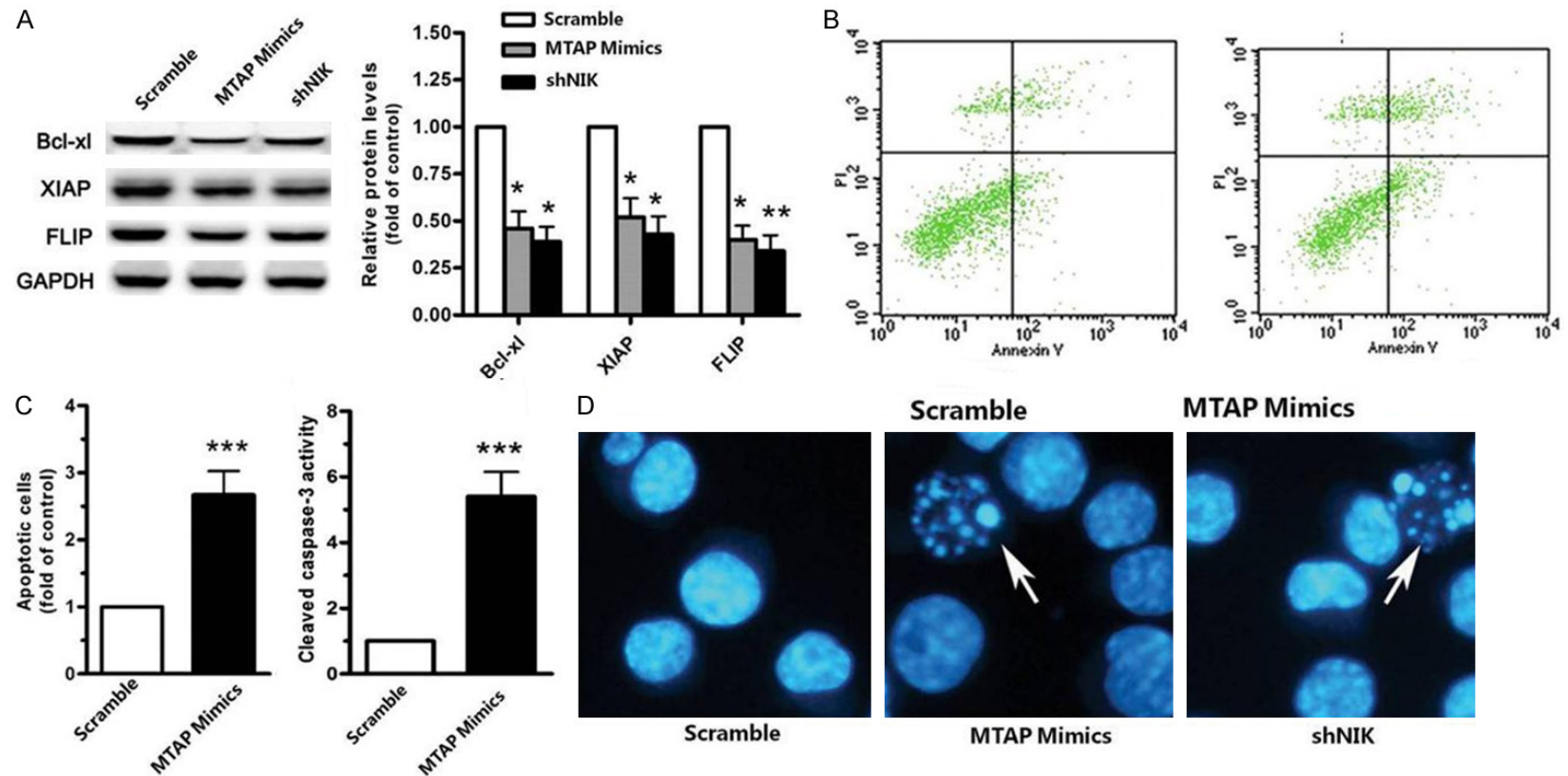


Figure 4. MTAP promotes cell apoptosis by inhibiting the NF- κ B pathway. A. Expression of NF- κ B related proteins; B. Quantitation of cell apoptosis by flow cytometry; C. Activity test of caspase 3; D. Immunofluorescence results of cell apoptosis. *means $P < 0.05$, **means $P < 0.01$, ***means $P < 0.001$.

of NIK for NF- κ B pathway is important for survival of CRC cell lines.

Discussion

For different types of tumors which include Hodgkin's lymphoma, breast cancer, prostate cancer, and colorectal cancer, activation of NF- κ B can significantly increase tumor cell proliferation and suppress cell death. Additionally, the NF- κ B pathway also plays an important role in the inflammation, innate immune system and lymphocyte development [16]. Therefore, the further understand of the conduction of NF- κ B pathway could make process in the mechanisms behind the development of different types of tumors like colorectal cancer. Evidence has shown the important role of gene expression in the development of cancers [17]. Several genes involved in transcription are involved in the tumor generation and metastasis in colorectal cancer. For example, it is proved that MTAP is significantly related to the generation of colorectal cancer [18]. However, the function of MTAP in colorectal cancer still needs further study. The current study is mainly focused on the role of MTAP in the cell proliferation of colorectal tumor cells. This study results exhibit that significant MTAP downregulation is found in all colorectal cancer cases which means the loss of MTAP is the prerequisite for the development of colorectal cancer. These results also show that the downregulation of MTAP is normal in colorectal cancer and MTAP can be used as the marker for the prognosis and metastasis of colorectal cancer.

The NF- κ B pathway is activated or inhibited through gene expression and thus many tumors have resistance to the current cancer therapy. MTAP shows potential therapeutic effects by regulating NF- κ B pathway. Although the NF- κ B pathway plays an important role in many cellular processes, however, targeting inhibition of related genes which can activate the NF- κ B pathway hasn't been deeply studied [19]. The former study has shown that the members of MTAP family can promote the connection of NF- κ B subunit and DNA. This study shows that the increase of RelA in nucleus which is closely related to the NF- κ B pathway can lead to the enhancement of both the activities of classical and non-classical NF- κ B pathways [20]. Additionally, the recent study has shown that inhibition of MTAP suppresses the activation of

NF- κ B pathway in HeLa cells, although they also found that MTAP can increase the NF- κ B pathway by downregulating the I κ B α [21]. Therefore, MTAP protein may have multi-factor functional mode and is able to regulate the function of the NF- κ B pathway at different stages whether it is cell type dependence or stimulator dependence. However, since the discovery of p50-p50 homologous dimer complex in colorectal tumor tissues, an important selectivity for the colonic tumor cell survival could be by recruiting BAG-1 though the p50-p50 homologous dimer complex. In addition, the regulation mode of MTAP for RelA is different in different colorectal cancer cell lines. For instance, in HCT116 cells, MTAP is mainly binding to the coding region to affect the stability of mRNA and then regulate the RelA, however, in the RKO cells, the RelA is regulated by MTAP by two mechanisms. The expression of MTAP can be regulated through multiple mechanisms in different phenotypes of colorectal cell lines. One current study has shown that MTAP inhibition is DNA methylation dependent [22]. However, in our study, in order to find out whether NF- κ B is involved in the MTAP regulated development of colorectal cancer, MTAP high expression and low expression LoVo cell lines were generated and analyzed for NF- κ B pathway activation. The NF- κ B Pathway was suppressed with high expression of MTAP and thus MTAP may negatively regulate the NF- κ B pathway. Furthermore, low expression of MTAP and increasing expression of NIK led to NF- κ B activation in CRC cells. In addition, the partial recovery of MTAP impairs the NF- κ B induced CRC cell proliferation. Based on the fact that NF- κ B pathway plays a key role in the regulation of transcription in both normal and tumor cells, discovering the functions of epigenetic regulatory factors and MTAP in the NF- κ B pathway will enhance our understanding of the molecular mechanisms in CRC cells. All these findings indicate that abnormal gene expression is related to the malignant phenotype which will provide important clues for the clinical study and contribute to the identification of therapeutic targets for colorectal cancer diagnosis.

In general, downregulation of MTAP leads to the activation of NF- κ B pathway and colorectal cancer phenotype. In addition, these results provide evidence that MTAP is an important tumor inhibitor.

Disclosure of conflict of interest

None.

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