Original Article

Inhibition of autophagy by 3-methyladenine promotes migration and invasion of colon cancer cells through epithelial mesenchymal transformation

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Background: Colon cancer is the third leading cause of tumor-related deaths in the world. Inhibition of autophagy in the treatment of malignant tumors has attracted extensive attention. However, the association between inhibition of autophagy by 3-methyladenine (3-MA) and epithelial mesenchymal transformation (EMT) in colon cancer cells has not yet been fully elucidated.

Methods: In this study, colon cancer cell lines (LOVO and SW620) were treated with 3-MA. Wound healing assays and transwell assays were used to detect the effect of inhibition of autophagy on the migration and invasion of colon cancer cells. The expression of EMT-associated markers, Twist1, E-cadherin, and vimentin, in colon cancer cells with and without 3-MA treatment was detected by Western blotting, immunohistochemistry, immunofluorescence staining, and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Results: Our data showed that inhibition of autophagy by 3-MA significantly enhanced the migration and invasion of colon cancer cells. At the molecular level, inhibition of autophagy upregulated the expression of Twist1 and vimentin, downregulated the expression of E-cadherin, and induced the EMT of colon cancer cells.

Conclusions: Inhibition of autophagy by 3-MA upregulated the expression of Twist1 in colon cancer cells and promoted cancer cell migration and invasion through EMT. Inhibition of autophagy may have adverse effects on colon cancer.

Keywords: Colon cancer; epithelial mesenchymal transformation (EMT); autophagy; Twist1

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Introduction

Colon cancer is one of the most common malignancies and is the third leading cause of cancer-related deaths worldwide (1,2). Despite significant progress in diagnosis and treatment, cancer metastasis remains the main cause of death. Cancer metastasis can be achieved by activating the epithelial mesenchymal transformation (EMT) process. EMT is characterized by the loss of tight junction protein E-cadherin, the expression of mesenchymal markers such as vimentin, and an increase in cell motility, which drives the cascade of invasion and metastasis (3-6).

Autophagy is an evolutionarily conserved catabolic

process that maintains the stability of the intracellular environment (7). In tumors, autophagy plays different roles in different cells and tissues. It can promote or inhibit the development of tumors (8,9). Microtubule-associated protein light chain 3B (MAPLC3B, hereafter referred to as LC3B) is a marker of the autophagy pathway. When autophagy is induced, LC3B-I is transformed into LC3B-II, and the ratio of LC3B-II/LC3B-I is related to the number of autophagosomes (10). In addition, autophagy plays a major role in the degradation of selective autophagy receptors and the signal regulatory protein SQSTM1/p62 (hereafter referred to as p62). Therefore, the conversion of LC3B-I into LC3B-II and the selective degradation of p62 are widely used as markers for monitoring autophagic activity (10).

There is much evidence demonstrating that inhibition of autophagy can improve cancer treatment. Early clinical trials have shown that lysosomal inhibitors, such as hydroxychloroquine, have a good anticancer effect by inhibiting autophagy (11), however, studies have found that inhibition of autophagy may have adverse effects in cancer treatment (11). It is also possible that different inhibitors exert different effects. 3-methyladenine (3-MA) can inhibit phosphatidylinositol 3-kinase (PI3K) activity and prevent the formation of autophagosomes and autophagic vacuoles (10). The effects of inhibition of autophagy by 3-MA on the EMT and metastasis of colon cancer cells are unclear.

The transcription factor Twist1 is a basic helix-loop-helix protein and is a core regulator of early embryonic morphogenesis and cancer development and metastasis (12,13). Twist1 results in loss of adhesion in epithelial cells by downregulating the expression of E-cadherin, thereby promoting EMT and cancer metastasis (3-6,12,13). There is currently a paucity of data on the effects of autophagy on Twist1 in colon cancer. This investigation examined the effects of inhibition of autophagy by 3-MA on Twist1 expression and cancer cell invasion in colon cancer cell lines, so as to provide theoretical support for potential colon cancer treatments. We present the following article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-1736/rc).

Methods

Tissue samples

A total of 56 paraffin-embedded colon cancer samples were obtained from the Department of Pathology at The Second Hospital of Tianjin Medical University. Clinical, demographic data, and pathology, including age, gender, primary site, metastatic site, and lymph node involvement, were collated from electronic medical records. Histologically, there were 42 cases of low-grade tumor and 14 cases of high-grade tumor. This study was approved by the Ethics Committee of The Second Hospital of Tianjin Medical University (No. KY2022K184) and conducted in accordance with the Declaration of Helsinki (as revised in 2013). Individual consent for this retrospective analysis was waived.

Immunohistochemical staining

Sections (4 μm thick) of routine formalin-fixed and paraffin-embedded tissues were deparaffinized, rehydrated, and subjected to antigen retrieval. Immunohistochemical staining was performed according to the manufacturer's recommendations with a fully automated system (Leica Biosystems, Bond-Max, Germany). Sections were stained with an anti-Twist1 antibody (25465-1-AP, Proteintech, USA). Appropriate positive and negative controls were used.

Cell culture and treatment

The human colon cancer cell lines LOVO and SW620 were obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured and passaged according to standard protocols. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) at 37 ℃ in a humidified incubator (SANYO, Tokyo, Japan) with 5% CO_2. For the *in vitro* experiments, cells were co-cultured with the autophagy inhibitor 3-MA (5 mM; MCE, USA) for 0, 24, and 48 hours. We had blank group and 3-MA group. Subsequently, the cells were prepared for the blank group and 3-MA group.

Wound healing assay

Colon cells treated with or without 3-MA were collected and seeded in 6-well plates. When the cells reached approximately 80% confluency, the bottom of the well was scratched uniformly using sterile pipette tips. After scratching, the cells were washed three times with phosphate buffered saline (PBS) and observed under light microscopy at 0, 24, and 48 hours to assess the width of the scratch line.

Transwell assays

Cells were seeded in Matrigel Invasion Chambers (EMD Millipore, Billerica, MA, USA) in a 24-well culture plate. After incubation for 24 or 48 hours at 37 ℃ in an atmosphere with 5% CO_2, noninvading cells within the insert chamber were removed, and the upper layer of the transwell was briefly wiped with a cotton swab. The bottom of the membrane was fixed with 4% paraformaldehyde for 30 minutes, stained with 0.1% crystal violet for 10 minutes, and then imaged. The invading cells on the membrane were counted under a bright-field microscope (Zeiss, Oberkochen, Germany).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from cells using TRIzol reagents (Invitrogen, MA, USA) according to the manufacturer's protocol. The Takara Reverse Transcription Kit (Takara, Japan) was used to synthesize the cDNA. A SYBR Premix ExTaq kit (Takara, Japan) and Thermal Cycler DiceTM Real Time System III (Takara, Japan) were used for RT-PCR. Expression levels in each sample were normalized to the expression level of β-actin. The $2^{-\Delta\Delta Ct}$ method was applied to analyze mRNA expression with β-actin as the normalization control. The following primer sequences were used: Twist1 forward 5'TCTACCAGGTCCTCC AGAGC3'; Twist1 reverse 5'CTCCATCCTCCAGACCGAGA3'; β-actin forward 5'CGTGACATTAAGGAGAAGCTG3'; β-actin reverse 5'CTAGAA GCATTTGCGGTGGAC3'.

Western Blot analysis

Cells were homogenized with RIPA lysis buffer (Sigma, MA, USA) to extract the protein content and the concentration was detected using the BCA Protein Quantitative Kit (Beyotime, Beijing, China). Protein samples were diluted in 5× sodium dodecyl sulfate (SDS) sample loading buffer. Equal amounts of protein (10 μg) in a 10 μL volume were separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, MA, USA). Membranes were incubated overnight at 4 ℃ with primary antibodies specific for E-cadherin (EP6, Zsbio, China), vimentin (EP21, Zsbio, China), p62 (18420-1-AP, Proteintech, USA), Twist1 (25465-1-AP, Proteintech, USA), light chain 3 (LC3; 18420-1-AP, Proteintech, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab53573, Abcam, UK). Thereafter, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Zsbio, Beijing, China) at 25 ℃ for 1.5 hours. Antibody solutions were diluted in 5% milk in Tris-buffered saline/Tween (TBS/T) buffer. Specific protein bands were visualized using an ECL substrate kit (Merck Millipore, MA, USA). The ImageJ software was used to determine the relative expression of the target protein to GAPDH.

Immunofluorescence staining

For immunofluorescence staining, cells were cultured on glass coverslips (24-well plates). The cells were washed with PBS, fixed with 4% paraformaldehyde for 20 minutes, and permeabilized with 0.2% Triton X-100 in PBS for 4 minutes. Subsequently, the cells were incubated overnight at 4 ℃ with the appropriate primary antibodies diluted in PBS containing 1% bovine serum albumin (BSA). The cells were washed 3 times and then incubated for 45 minutes with FITC-conjugated donkey anti-rabbit secondary antibodies diluted in the same buffer. Imaging was performed using a fluorescence microscope (Olympus, Japan).

Gene Expression Profiling Interactive Analysis (GEPIA)

The GEPIA platform (http://gepia2.cancer-pku.cn/#index) is an interactive web server for cancer expression profile data which integrates gene expression profile data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEX) projects. Data related to the transcriptional expression of Twist1 in colon cancer tissues and normal tissues were downloaded from GEPIA.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 software (La Jolla, CA, USA). Student's *t*-tests were performed on samples that passed the Shapiro-Wilk normality test and variance homogeneity test. Nonparametric Wilcoxon signed-rank tests were performed for samples that did not exhibit a normal distribution or variance unequal. Data are presented as the mean ± standard deviation (SD). A P value <0.05 was considered statistically significant. All experiments were technically repeated 3 times, and the results were taken as the average.

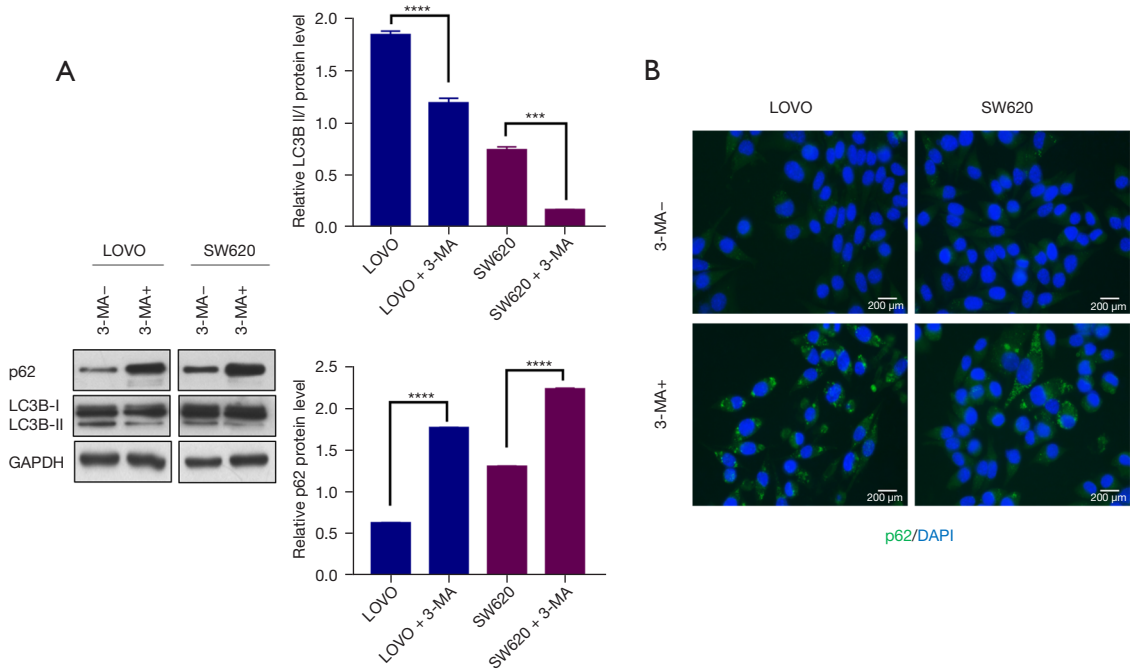

Figure 1 Treatment with 3-MA inhibited autophagy in colon cancer cells. (A) The protein levels of LC3B II/I and p62 were measured in LOVO and SW620 cells with and without 3-MA treatment using Western blot analysis. (B) Immunofluorescence staining of p62 in the above treated colon cancer cells. Scale bar =200 μm. ****, P<0.0001; ***, P<0.001. 3-MA, 3-methyladenine; LC3B, microtubule-associated protein light chain 3B; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Results

Inhibition of autophagy in colon cancer cells by 3-MA

Western blotting analysis showed that LOVO and SW480 cells treated with 3-MA had significantly decreased ratios of LC3B-II/LC3B-I and significantly elevated levels of p62 compared to untreated cells (*Figure 1A*). Immunofluorescence staining showed much more p62 accumulation in cells treated with 3-MA (*Figure 1B*), indicating that 3-MA effectively inhibited autophagy.

Inhibition of autophagy by 3-MA promotes the migration and invasion of colon cancer cells

To investigate whether the inhibition of autophagy by 3-MA has an effect on the function of colon cancer cells, the migration and invasion abilities of colon cancer cells before and after 3-MA treatment were investigated. Wound healing assays showed that cells treated with 3-MA migrated at a significantly faster rate in the leading edge of the scratch compared with untreated control cells (*Figure 2A*). Similarly, transwell assays showed that the number of cells per field markedly increased after 3-MA treatment (*Figure 2B*). This suggested that inhibition of autophagy by 3-MA promoted the migration and invasion of colon cancer cells.

Inhibition of autophagy by 3-MA increases vimentin expression and decreases E-cadherin expression in colon cancer cells

To further investigate whether the inhibition of autophagy by 3-MA triggered the EMT process, the expression of EMT-related proteins was evaluated. Western blotting analysis showed that the expression of epithelial marker E-cadherin was significantly decreased in colon cancer cells treated with 3-MA compared with untreated cells (*Figure 3A*). However, the expression of the mesenchymal marker vimentin was markedly upregulated in colon cancer cells treated with 3-MA (*Figure 3B*). These results suggested that autophagy inhibition changed the colon cancer cell traits and induced EMT.

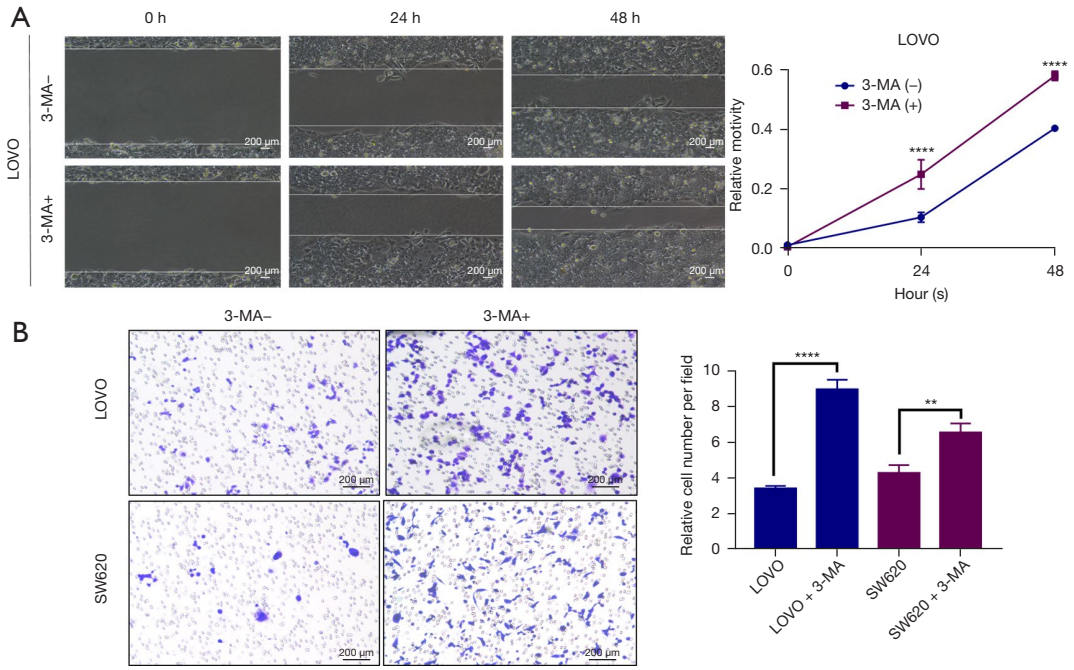

Figure 2 Inhibition of autophagy by 3-MA promoted the migration and invasion of colon cancer cells. (A) The wound healing test showed that the migration ability of colon cancer cells treated with 3-MA was significantly enhanced compared with untreated colon cancer cells. (B) The transwell assays showed that the relative number of cells per field was markedly increased in cells treated with 3-MA compared with untreated cells. Stained with 0.1% crystal violet. Scale bar =200 μm. ****, P<0.0001; **, P<0.05. 3-MA, 3-methyladenine.

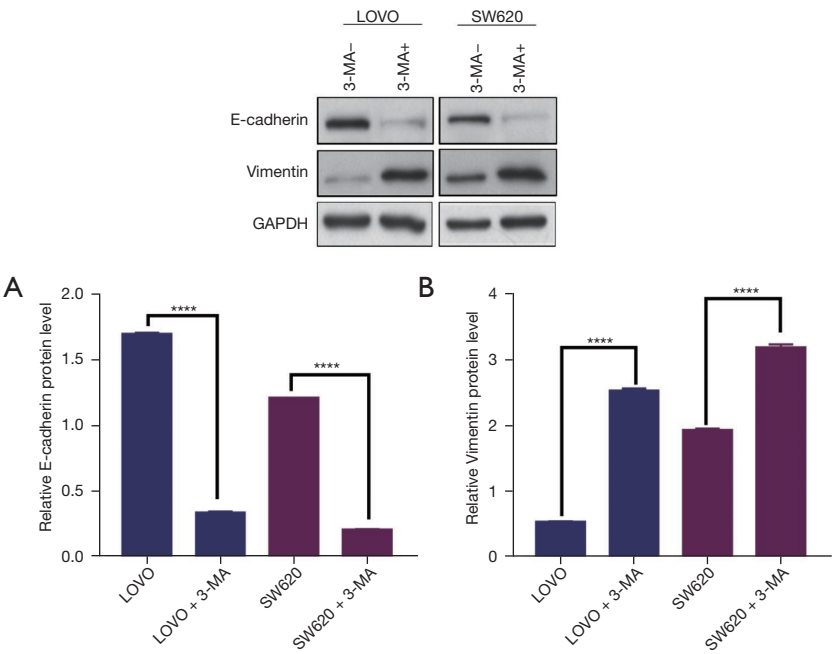

Figure 3 Inhibition of autophagy by 3-MA induced EMT in colon cancer cells. (A) Western blotting analysis showed that the expression of E-cadherin was significantly decreased compared with untreated colon cancer cells. (B) Cells treated with 3-MA showed markedly increased expression of the vimentin compared with untreated cells. ****, P<0.0001. 3-MA, 3-methyladenine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMT, epithelial mesenchymal transformation.

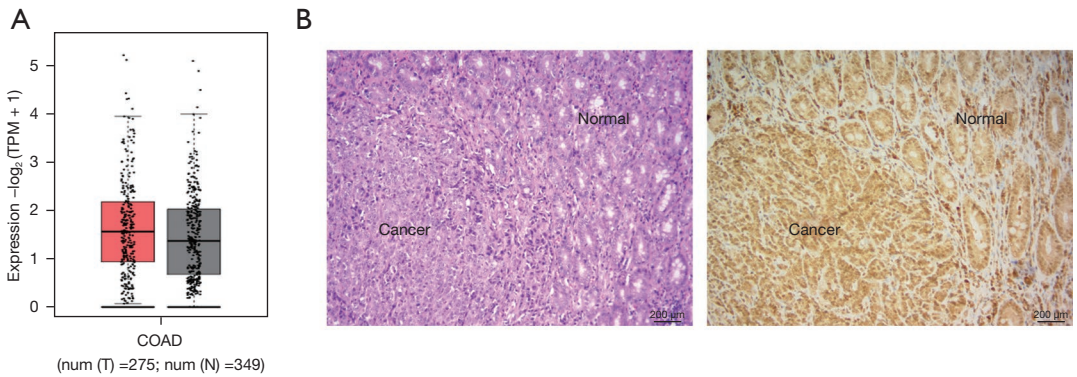

Figure 4 The expression of Twist1 in tumor and normal tissues of colon cancer patients. (A) Analysis of the GEPIA data showed that the expression of Twist1 in tumor tissues was slightly higher than that in normal tissues, but there was no significant difference. (B) The expression of Twist l in normal and colon cancer tissues is showed by immunohistochemical staining. Scale bar =200 μm. TPM, transcripts per kilobase million; COAD, colon adenocarcinoma; T, tumour; N, normal; GEPIA, Gene Expression Profiling Interactive Analysis.

The expression of Twist1 in tumor tissues and normal tissues of colon cancer

To clarify the mechanisms involved in the regulation of EMT by inhibiting autophagy, the expression of Twist1 in colon cancer tissue and cells was examined. The GEPIA platform was used to analyze the expression of Twist1 in tumor tissues and normal tissues of colon cancer patients. The results showed that the expression of Twistl increased slightly in tumor tissues, but there was no significant difference between tumor tissues and normal tissues (*Figure 4A*). Furthermore, immunohistochemistry staining revealed that Twist1 was expressed in both normal and colon cancer tissues, and the staining range and intensity were comparable (*Figure 4B*). These results are consistent with the GEPIA analysis.

Inhibition of autophagy by 3-MA increases Twist1 expression in colon cancer cells

In vitro studies revealed that the mRNA (*Figure 5A*) and protein (*Figure 5B*) expression of Twist1 increased significantly in response to autophagy inhibition. The upregulation of Twist1 expression occurred in parallel with a decrease in E-cadherin expression and an increase in vimentin expression. Immunofluorescence staining showed higher Twist1 expression in cells treated with 3-MA compared to untreated cells (*Figure 5C*), suggesting that inhibition of autophagy promoted EMT by upregulating the expression of Twist1.

Discussion

This study demonstrated that inhibition of autophagy by 3-MA induced EMT in colon cancer cells. The role of autophagy in EMT has been widely reported, but the results are controversial. Several studies have shown that autophagy promotes EMT. For example, Alizadeh *et al.* (14) demonstrated that autophagy positive regulation of transforming growth factor (TGF)-β1 induced EMT in non-small cell lung cancer cell lines. Similarly, Dash *et al.* (15) reported that autophagy promoted EMT and metastasis by regulating the level of reactive oxygen species in hepatocellular carcinoma cells. However, recent investigations on different types of cancer have reported otherwise. Qiang *et al.* (16) demonstrated that autophagy mediates p62-dependent Twist1 degradation and promotes cell proliferation and migration in autophagy-related gene (ATG) knockout mouse embryonic fibroblasts and human squamous cell carcinoma cells. In addition, autophagy-dependent SNAI1 degradation inhibits EMT and metastasis of cancer cells in lung cancer and cervical cancer cell lines (17). Taken together, these studies suggest that the role of autophagy in EMT may have tissue heterogeneity, or produce different effects under different conditions.

The results of the few studies examining the role of autophagy in EMT of colon cancer cells remain controversial. For example, suppression of beclin-1 (a key molecule involved in autophagy activation) expression through gene knockout significantly reduced EMT and invasive behaviors in colon cancer cells (18). However,

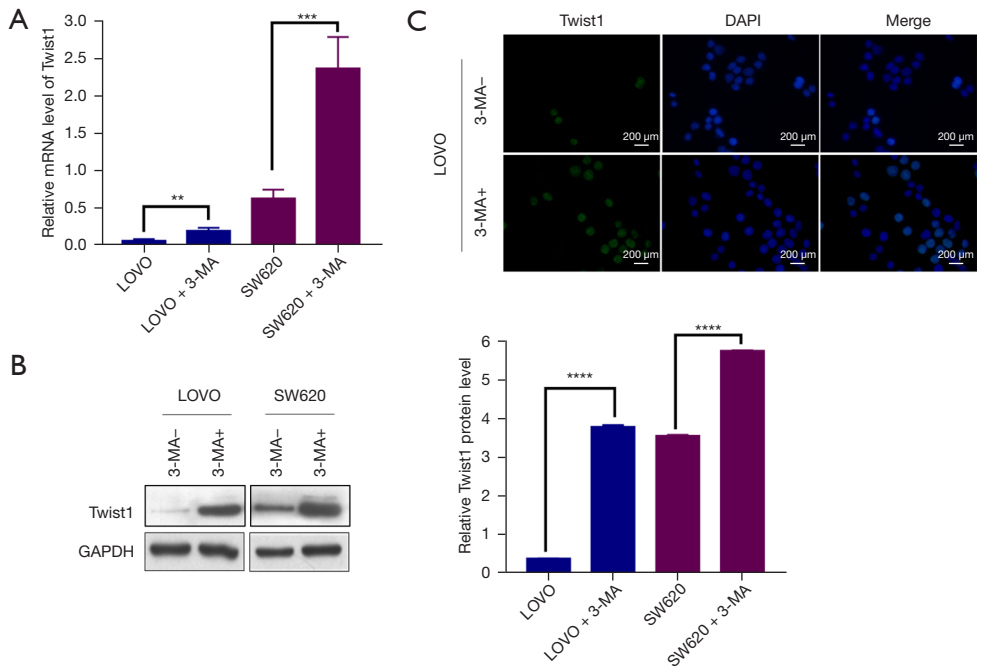

Figure 5 Inhibition of autophagy by 3-MA increased Twist1 expression in colon cancer cells. (A) The mRNA and (B) protein expression of Twist1 was significantly elevated in response to autophagy inhibition. (C) Immunofluorescence staining of Twist1 in LOVO cells with and without 3-MA treatment. ****, P<0.0001; ***, P<0.001; **, P<0.05. Scale bar =200 μm. 3-MA, 3-methyladenine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

according to an updated report by Islam Khan *et al.* (19), RAMS11 promotes colorectal cancer through mTOR-dependent inhibition of autophagy and promotion of EMT. In this study, we found that inhibition of autophagy by 3-MA decreased the expression of E-cadherin, increased the expression of vimentin, and promoted the migration and invasion of colon cancer cells. These results suggested that there is a close correlation between autophagy and cell phenotype or the EMT process in colon cancer. In addition, inhibition of autophagy resulted in p62 accumulation and upregulation of the EMT-related transcription factor Twist1. Studies have shown that p62 contains domains that interact with many signal activators (20). P62 accumulated during autophagy deficiency blocks the degradation of Twist1 through the interaction between its ubiquitin related domain and Twist1, and then regulates the expression of E-cadherin, induces EMT, and promotes tumor metastasis (21,22). Based on our data, we speculate that the EMT process of colon cancer might follow this regulatory pathway.

It is noteworthy that there was no significant difference in the expression of Twist1 in normal tissues and tumor tissues of colon cancer patients as analyzed using data from the GEPIA database. The Twist1 protein is an unstable protein regulated by the ubiquitin proteasome system through F-box protein and E3 ubiquitin ligase (23). It is degraded by autophagy and other pathways, such as the proteasome system, and can change dynamically *in vivo*. In addition, the expression and cellular localization of Twist1 drives the resultant downregulation or loss of E-cadherin expression, and for Twist1 to function as an activator and/or repressor of the transcription of a target gene, efficient nuclear localization is essential (24). Further research is required to clarify the intracellular localization of Twist1 in colon cancer tissue, so as to understand its functional role.

There is much evidence in the literature and clinical trials showing that autophagy inhibitors combined with chemotherapy or targeted therapy will benefit cancer patients (11,25). This study demonstrated that inhibition of autophagy by 3-MA promoted the migration and invasion of colon cancer cells, suggesting that inhibition of autophagy may have adverse effects on colon cancer, at least under certain conditions. In addition, Twist1 is a key downstream regulator of p62. It is speculated that targeting p62-

mediated Twist1 stabilization may be a promising cancer prevention and treatment strategy.

In conclusion, this investigation demonstrated that inhibition of autophagy upregulates the expression of Twist1 in colon cancer cells and promotes cancer cell migration and invasion through EMT. Furthermore, inhibition of autophagy may have adverse effects on colon cancer.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-1736/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-1736/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-1736/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The Second Hospital of Tianjin Medical University (No. KY2022K184) and individual consent for this retrospective analysis was waived.

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