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E-cadherin inhibits the proliferation and migration of human colorectal cancer cells through Hippo signaling pathway

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ABSTRACT

E-cadherin (E-cad) is a crucial regulatory factor in rescue Epithelial-mesenchymal transition and is involved in the occurrence of various malignant tumor. However, the mechanisms by which E-cadherin regulates tumor metastasis in CRC remain unclear. We established sh-E-cad (silenced by short hairpin RNA) and rescue-E-cad (overexpressed by E-cad plasmid transfection) CRC cell lines to investigate the role of E-cad in CRC *in vitro.* Immunohistochemistry, clonogenic assays, scratch wound healing assays, CCK-8 assays, flow cytometry, Transwell assay, real time-PCR and Western blot were employed to investigate the underlying mechanisms by which E-cad involve the progression of CRC. In CRC tissues, E-cad expression was significantly reduced, while YAP expression was markedly elevated. Silencing E-cad induced a significant increase of clonogenic ability in CRC cells, which was reduced upon rescue of E-cad expression. Transwell assays indicate that low expression of E-cad enhances the cell migration, a finding corroborated by scratch wound healing experiments. CCK-8 results demonstrate that silencing E-cad promotes the proliferation of CRC cells. Importantly, we found that E-cad influences apoptosis rather than the cell cycle. Analysis of Hippo signaling pathway-related factors revealed that silencing E-cad resulted in significantly decreased expression of MST1/2 and LATS1/2, as well as reduced phosphorylation levels of YAP, while YAP expression was significantly increased. Additionally, immunofluorescence confirmed the nuclear translocation of YAP. Our study indicates that E-cad regulates the malignant progression of CRC *via* the Hippo signaling pathway, offering a potential new strategy for CRC treatment.

Key words: colorectal cancer; E-cadherin; Hippo signaling pathway; apoptosis.

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Introduction

Colorectal cancer (CRC) is one of the three major malignancies in the world, which has caused serious impacts on human health. A CRC statistics in 2024 estimates that approximately 153000 new cases and 52000 death cases are predicted to occur in the United States.[1] Another epidemiological investigation shows that there are approximately 592000 new cases and 309000 death cases due to the colorectal malignancy in China.[2] For early-stage patients 5-year survival rates is nearly 61%, while this number will be less than 40% in advanced CRC.[3,4] In patients with CRC, liver and lung metastasis is the primary cause of deaths.[5,6] It was reported that approximately 10-18% of rectal cancer patients and 5-6% of CRC patients have pulmonary metastases.[7] Metastasis has become an important factor seriously affecting the prognosis of patients suffering from CRC.

Epithelial-mesenchymal transition (EMT) has been shown to be a pivotal part in cancer initiation and progression, with its activation primarily regulated by transcription factors including E-cadherin (E-cad), Snail family transcriptional repressor 1 and zinc finger E-box-binding homeobox 1.[8] Numerous research showed that E-cad is an indicator of unfavorable prognosis in different cancer. In ovarian cancer, patients with membrane-expressed E-cad have a longer survival time than those without E-cad expression at advanced stages.[9] In animal model of breast cancer, E-cad interacts with epidermal growth factor receptor and leads to hyper-activation of ERK.[10] Similarly, low-expression of E-cad in tissues of patients with endometrial carcinoma also with poor prognosis.[11] However, how the E-cad influence the tumor cell invasion in CRC was unclear.

The Hippo pathway is proved to be a key factor in many biological progresses when it was discovered 20 years ago.[12] Animal study suggests that the Hippo pathway serves its function with a huge amount of proteins such as mammalian sterile 20-like kinase 1/2 (MST1/2), large tumor suppressor kinase 1/2 (LATS1/2), Yes-associated protein 1 (YAP) and WW-domain-containing transcription regulator 1.[13] Increasing experiments have clarified the underlying mechanism of Hippo pathway in CRC. Researchers found activating Hippo/YAP pathway can mediate the beneficial effect of resveratrol on proliferation and apoptosis in human colonic cancer cells.[14] In addition, the anti-tumor capability of Wogonin is based on IRF-3-mediated Hippo pathway, which could be reversed by the YAP1 overexpression.[15] Mechanistically, how Hippo pathway impact the EMT and the modulating way of E-cad on Hippo pathway in CRC was not clear.

In this study, we aim to investigate the regulation of E-cad and its interaction with EMT with the CRC progression. We found that E-cad improves cell migration through the Hippo signaling pathway. E-cad Knockdown enhanced EMT and tumor cell migration in colorectal cancer. Importantly, our findings provide new strategy of leveraging the potential therapeutic target of E-cad for the invasive and metastatic CRC.

Materials and Methods

Participants

We collected the tumor tissues of 60 colon cancer patients (38 males and 22 females) admitted to the First Affiliated Hospital of Guangxi University of Chinese Medicine from January 2020 to June 2021. The patients were aged 20-87 (median: 64 years-old). All the specimens were confirmed as colorectal adenocarcinoma by biopsy and no loss of mismatch repair protein was detected by immunohistochemistry, and no chemotherapy was performed to patients. This study follows the «Helsinki» Declaration and is approved by the Ethics Committee of First Affiliated Hospital of Guangxi University of Chinese Medicine (Approval no. GXZYYSYJS-2023-014-01). All participating patients provide written consent form.

Immunohistochemistry assay

The tissues of 60 patients with CRC were acquired and fixed in 10% neutral buffered formalin for 24 h. After gradient dehydration and clearing, tissues were infiltrated and immersed with paraffin for twice, each for 30 min, embedded in molten paraffin and cut into sections (5 μm). In immunohistochemistry (IHC) experiment, CRC tissues were processed with deparaffinization and rehydration, then washed in deionized water. The intrinsic peroxidase activity and nonspecific antibody binding sites were blocked with Novolink polymer detection system. The sections were incubated at 4°C with the primary antibodies to E-cad (1:200, ab231303; Abcam, Cambridge, UK) and YAP1 (1:200, ab52771; Abcam) overnight. After incubation at room temperature with an enhancer for 20 min, the sections were sequentially incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (BS-0295G-HRP, Bioss Technology Co., Ltd., Beijing, China) for 1 h. A DAB substrate (ZLI-9017, Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) and hematoxylin were used to complete the color reaction and counterstaining. Finally, the sections were dehydrated through gradient ethanol and imaged using a microscope (Olympus BX53). For negative control, all the procedures were same as other groups except the omission of primary antibody. For the positive control, all the purchased antibodies and reagents were already proved to be high effective in published articles and the researchers strictly followed the experiment protocol.

Cell lines

HCT116, SW480 cells were selected for *in vitro* study due to their different biological characteristics of early lesions and late-stage metastatic tumors, while 293T cells were used for control experiment. The cell lines were provided by American Type Culture Collection (Manassas, VA, USA). 293T and SW480 were cultured in DMEM (Cat. 11995065; Gibco, Beijing, China) medium added with 10% fetal bovine serum (Cat. FSD500; Excell Bio, Shanghai, China). HCT116 was maintained in McCoy's 5A (Cat. PM150710; Pricella Biotechnology Co., Ltd., Wuhan, China) medium with 10% fetal bovine serum. The incubation condition was set to be at 37°C and 5% CO_2.

Lentivirus transduction and generation of stable cell lines

E-cad human-tagged CDS clone lentiviral particle was procured from EnvirusTM (Rockville, MD, USA). The E-cad targeted short hairpin RNA (shRNA) and non-eGFP-luciferase plasmid were purchased from the Sangon Biotech (Sangon, China). The target sequence of the E-cad shRNA is 5'- ATTTTAAAGGT-TAAAGTAGCCTTCAAGAGAGGCTACTTTAACCTT-TAAAAT -3'. 293T cells were transfected with the empty vector, and E-cad shRNA for 24 h to make the lentiviral particles. The lentivirus pLKO.1 with shRNA construct-E-cad shRNA expression vector virus solution was applied to the cells of six-well plates for 8 h according to grouping to infect, and polybrene was added to enhance the virus transfection effect. The virus solution was replaced with complete medium for 24 h, and the cells were selected in complete medium with PURO to obtain a cell line with stable E-cad knockdown. The cells that received sh-NC vector transfection were set to be the negative control, the Sh-E-cad group were conditioned with sh-E-cad vector transfection, and the

cells in E-cad rescue group were transfected with E-cad overexpression plasmid after constructing E-cad knockdown stable transformation strain.

CCK-8 testing

HCT116 and SW480 were seeded in 96-well plate (2000 cells per well). After incubating for 6 h, intervention treatment is performed on the cells according to experimental grouping, with 6 wells set up in each group. 2 h before the end of the experimental intervention, 10 μL CCK8 solution per well was added and continue to incubate for 2 h. After incubation, the plate was tested at 450 nm using a spectrophotometer.

Clone formation testing

HCT116 and SW480 cells were incubated for 2 weeks (2000 cells per well). After that, the cells were undergone 20 min fixation in 4% paraformaldehyde. 0.2% crystallization purple staining was added for 5 min. Eventually, the plate was placed in room temperature overnight and the clone formation rate was calculated.

Transwell assay

Fifty (50) μL BD gel (Cat. 354230; Corning Inc., Corning, NY, USA) was diluted with serum-free medium (VMatri gel: V medium=1:4) and spread in Transwell chamber. the chamber was placed in a 37°C incubator for 6 h. Cells (20000 cells per well) were plated in the upper chamber of Transwell inserts (Cat. 3413; Corning Inc.) for 24 h and received 4% formaldehyde fixation for 15 min in sequence, then using 0.05% crystal violet for cell staining. The cells were harvested and prepared for the imaging analysis of the migrated cells. This experiment was performed in triplicates and repeatedly analyzed by Image-Pro Plus 7.0 more than three times.

Wound healing testing

HCT116 and SW480 cells were plated in 6-well plates (1000000 cells per well) and incubation for 24 h. The scratches caused by the perpendicular pipette tip will occur when the cell density reaches 90%. The effect of E-cad of wound healing was visualized after washing with PBS by the microscope at 0 and 24 h. The area was captured and calculated with ImageJ. All experiment were performed in triplicates and repeatedly analyzed more than three times.

Flow cytometry

To evaluate cell cycle, the cells were fixed in 75% alcohol overnight and were incubated for 30 min in 500 μL of Propidium Iodide (PI)/RNase staining solution. Red fluorescence was detected at 488 nm to assess the cell cycle. Cells that fall falling within G0/G1, S and G2/M are counted and divided by the total number of cells to get the percentage for each phase.

Fluorescein (FITC) annexin V apoptosis detection kit (C1052; Beyotime Biotechnology, Shanghai, China) is used for apoptosis evaluation according to the manufacturer's instruction. The cells were resuspended and incubated in binding buffer supplemented with Annexin V FITC and PI for 15 min. Then 400 μL binding solution was added. The apoptosis was analyzed by flow cytometer (Attune NxT; Thermo Fisher Scientific, Waltham, MA, USA).

Real-time PCR

A cell/tissue total RNA isolation kit V2 (RC112) was used for total RNA extraction. The extracted RNA was reverse transcription to cDNA by HiScript lll first Strand cDNA synthesis kit (R312). The primer sequences were listed below: E-cad, forward: TTTGAGGTCTCTCTCACCAC, reverse: GTAGCTATGATTAGGGCTGTG, MST1, forward: AACTGAAACGCCAGGAATCC, reverse: TTCCAGACTGCTGTTCTTGG, MST2, forward: ATGAGGAACAGCAACGAGAATTGG, reverse: GTCTCCATCTTGAGGAACTTTC, LATS1, forward: CCTAATCAAGGACAGAGAGG, reverse: ATATGACGAAGGAGCAGCAG, LATS2, forward: CAAGAAATGGCCAAAGCTGG, reverse: CGGTTCAGGACATCCTTTTTC, YAP, forward: AATCCCAGCACAGCAAATTCTC, reverse: TTAGTCCACTGTCTGTACTCTC, GAPDH, forward: GAAAGCCTGCCGGTGACTAA, reverse: GCATCACCCGGAGGAGAAAT. Real-time PCR analysis was performed using Taq Pro Universal SYBR qPCR Master Mix (Cat. Q712; Vazyme, Nanjing, China) and a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot

The target cells were placed on ice and lysed with RIPA buffer (Cat. R0020; Solarbio, Beijing, China) supplemented with phosphatase inhibitors and protease inhibitors. The denaturized protein samples were loaded onto 7.5% sodiumlauryl sulfate polyacrylamide gel for electrophoresis and transblotted to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in TBST. Then the membranes were incubated overnight at 4°C with primary antibodies and for 1 h at room temperature with secondary antibodies. Primary antibody: anti-E-cad antibody (Cat. ab231303, 1:1000; Abcam), anti-p-YAP1 antibody (Cat. ab76252, 1:10000; Abcam), anti-YAP1 antibody (Cat. ab52771; 1:5000; Abcam), anti-Histone H3 antibody (Cat. Ab1791; 1:5000 Abcam) as the internal reference for the experiment of nuclear translocation, and anti-GAPDH antibody (Cat. ab8245, 1:10000; Abcam) as the internal reference for E-cad, YAP1 and p-YAP1. Secondary antibodies: goat anti-rabbit or goat anti-mouse. The analysis was performed with chemiluminescence imaging instrument (JP-K6000).

Immunofluorescence

The cells were fixed with 4% paraformaldehyde (#P1110; Solarbio) and permeabilized in 0.5% Triton-x-100, respectively. Then the cells were blocked with 5% BSA at room temperature for 1 h and were incubated at 4°C for 24 h with anti-E Cadherin antibody (Cat. ab231303, 1:50; Abcam) and anti-YAP1 antibody (Cat. ab52771, 1:100; Abcam). The cells were incubated in fluorescent secondary antibody Alexa Fluor 488 (Cat. A-11066, 1:2000; ThermoFisher) or Alexa Fluor 594, respectively (Cat. Ab150080, 1:500; Abcam), and DAPI was added for staining nucleus. For negative control, cells were incubated in PBS without primary antibodies. For positive control, all the reagents used in this experiment were commercial product and already proved to be high effective according to the published data and the experiment protocol were strictly followed. Images were acquired by a confocal microscope (Leica TCS SP8). Co-localization ratio is calculated by overlap coefficient according to Colocalization Threshold using Fiji (Ver. 2.0.0).

Nuclear and cytoplasmic separation

Cells were washed twice with cold PBS. Two-hundred (200) μL of cytoplasmic protein extraction reagent A containing PMSF was added, and vortexed vigorously for 5 s to suspend the pellet, followed by an ice incubation for 10 min. Then, 10 μL of reagent B were add, vortexed again, and incubated on ice for 1 min; and, after another brief vortex, centrifuged at 4°C for 5 min. The supernatant was transferred to a tube for cytoplasmic protein, then removed and 50 μL of nuclear extraction reagent with PMSF were added. On following, vortexed every 2 min for 30 min on ice and centrifuged at 4°C for 10 min; the supernatant was transferred to a tube and 5×loading buffer was added and heated in boiling water for 10 min to denature the nuclear protein. Samples were stored at -20°C. The expression of extracted nuclear protein was detected as

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the procedure described in Western blot to verify the nucleoplamic separation.

Statistical analysis

GraphPad Prism (version 8.0) and R (version 4.2.1) were applied for statistical analysis. A Student's *t*-test was used to analyze the difference between two different groups. More than two groups of data were analyzed using one-way ANOVA followed by *post-hoc* Tukey's test. A *p*-value <0.05 was considered statistical significance.

Results

Expressions of E-cad and YAP in CRC patients

We analyzed 60 patient tumor and adjacent tissues with CRC by using IHC. The IHC result showed that the tumor tissues had a lower expression of E-cad compared with adjacent, while had a higher expression of YAP compared with adjacent tissues (Figure 1). This data implied that E-cad and YAP as key targets involved the progression of CRC.

E-cad induces EMT of CRC cells *in vitro*

We constructed CRC cell lines that silenced E-cad to study the effect of E-cad *in vitro*. Notably, the protein level of E-cad in CRC cells was significantly reduced after silencing, while after rescuing E-cad, the low expression E-cad was reversed (Figure 2 A,B). The results of CCK-8 assay showed that the proliferation ability of cells increased after silencing E-cad, while decreased after reverting to E-cad (Figure 2C). When the expression of E-cad decreased, the clonogenic abilities of SW480 and HCT116 cells were significantly increased (Figure 2D). What's more, the colonogenesis of the cells were partially reduced after reverting to E-cad. Similarly, we have reached a consistent conclusion in wound healing assay that low expression of E-cad significantly increases the CRC cell migration (Figure 2E). The results of the Transwell experiment showed that silencing E-cad also promoted the tumor cell invasion, which could be partially weakened by the rescue experiment (Figure 2F). These results suggest that E-cad, as a tumor suppressing factor, inhibited the malignant phenotype of CRC cells.

E-cad inhibits the cell apoptosis of CRC *in vitro*

The result of flow cytometry indicates that no remarkable difference was found in the cell cycle distribution between the two types of cultured cells (Figure 3A). Subsequently, we explored whether the E-cad impact on the apoptosis. Interestingly, the apoptosis assay showed that knock-down E-cad could attenuate the apoptosis cell ratio both HCT116 and SW480 (Figure 3B). In summary, E-cad could influence the cell apoptosis and not cell cycle.

E-cad suppresses apoptosis of CRC cells *via* stimulating the Hippo signaling system

Based on the above results, we confirmed that E-cad (a classic marker of EMT) is essential for CRC progression and influences tumor cell apoptosis. Since Hippo signaling pathway participates the regulation of cell proliferation and apoptosis, we assumed that E-cad might act on the occurrence of colon cancer *via* Hippo signaling pathway. To test this hypothesis, we performed Western blot and found that the expression of MST1/2, LATS1/2, and the phosphorylation levels of YAP were significantly decreased due to the low expression of E-cad, while the expression of MST1/2, LATS1/2, and phosphorylation of YAP were increased after the restoration of E-cad (Figure 4A). Similarly, the real-time PCR results indicated that the levels of MST1, MST2, LATS1 and LATS2 were significantly reduced in the sh-E-cad group (Figure 4B).

E-cad upregulates the nuclear translocation of YAP

The nuclear translocation of YAP was verified by nucleoplasmic separation and Western Blot experiments (Figure 5A). We found that he nuclear translocation of YAP was notably elevated when the E-cad expression is silenced. Interestingly, the nuclear translocation of YAP was significantly reduced by E-cad rescue. The co-localization of E-cad and YAP was detected by fluorescent

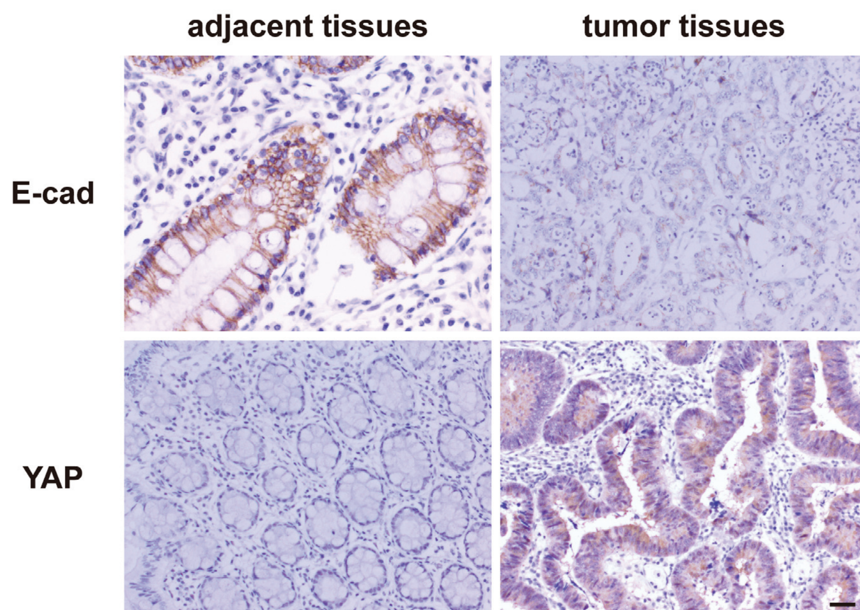

Figure 1. E-cad is low-expressed and YAP is over-expressed in CRC patients and correlated with unfavorable prognosis. The expression levels of E-cad and YAP in immunohistochemistry-stained tumor issues were analyzed. Scale bar: 100 µm.

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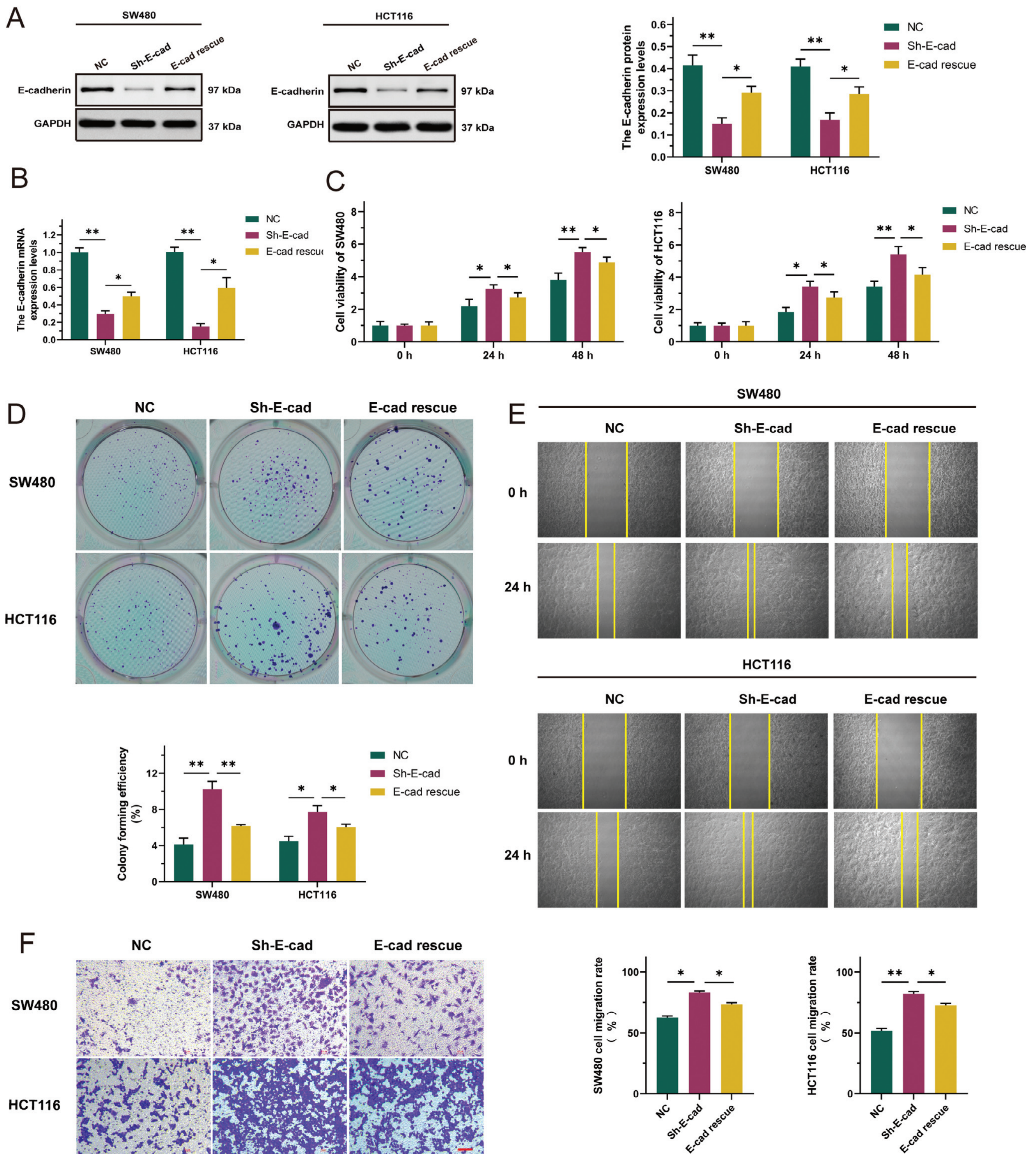

Figure 2. E-cad promotes EMT in CRC cells. **A**) The expression level of E-cad in HCT116 cells and SW480 cells transfected with control shRNA or specific E-cad shRNA for 24 h. **B**) The RNA levels of E-cad in HCT116 cells and SW480 cells transfected with control shRNA or specific E-cad shRNA for 24 h. **C,D**) Cell proliferation was tested through CCK-8 assay and clone formation assays. **E,F**) Cell migration was assessed through wound healing and migration assays. Scale bar: 50 µm. Data are presented as mean ±SEM (n=6 per group). *p<0.05, **p<0.01.

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double staining (Figure 5B). The result shows that the fluorescence intensity of E-cad and YAP in the Sh-E-cad group (E-cad knockdown group) was significantly reduced than control group. Similarly, the fluorescence intensity of E-cad and YAP in the E-cad rescue group was significantly increased.

Discussion

CRC characterized by the malignant tumor invasion in the colon or rectum, typically originating from polyps within the colon. It is one of the most common cancers worldwide, particularly in Western countries, where its incidence and mortality rates are notably high.[16] Here, we found that in tumor tissues, E-cad expression was significantly reduced, while YAP expression was markedly elevated. Silencing E-cad results in a significant increase of proliferation and migration of CRC cells, which was reduced under the rescue of E-cad expression. Importantly, we found that E-cad influences apoptosis rather than the cell cycle, which was correlated the Hippo signaling pathway with a significantly decreased expression of MST1/2 and LATS1/2, as well as reduced phosphorylation levels of YAP after silencing E-cad, while YAP expression was significantly increased. E-cad also was confirmed to upregulate the nuclear translocation of YAP. Our findings not only clarify the importance of Hippo signaling pathway in CRC development, but might offer a therapeutic strategy that targeting the hippo signaling pathway *via* E-cad regulation for more effective and personalized CRC treatment including monotherapy or combination with

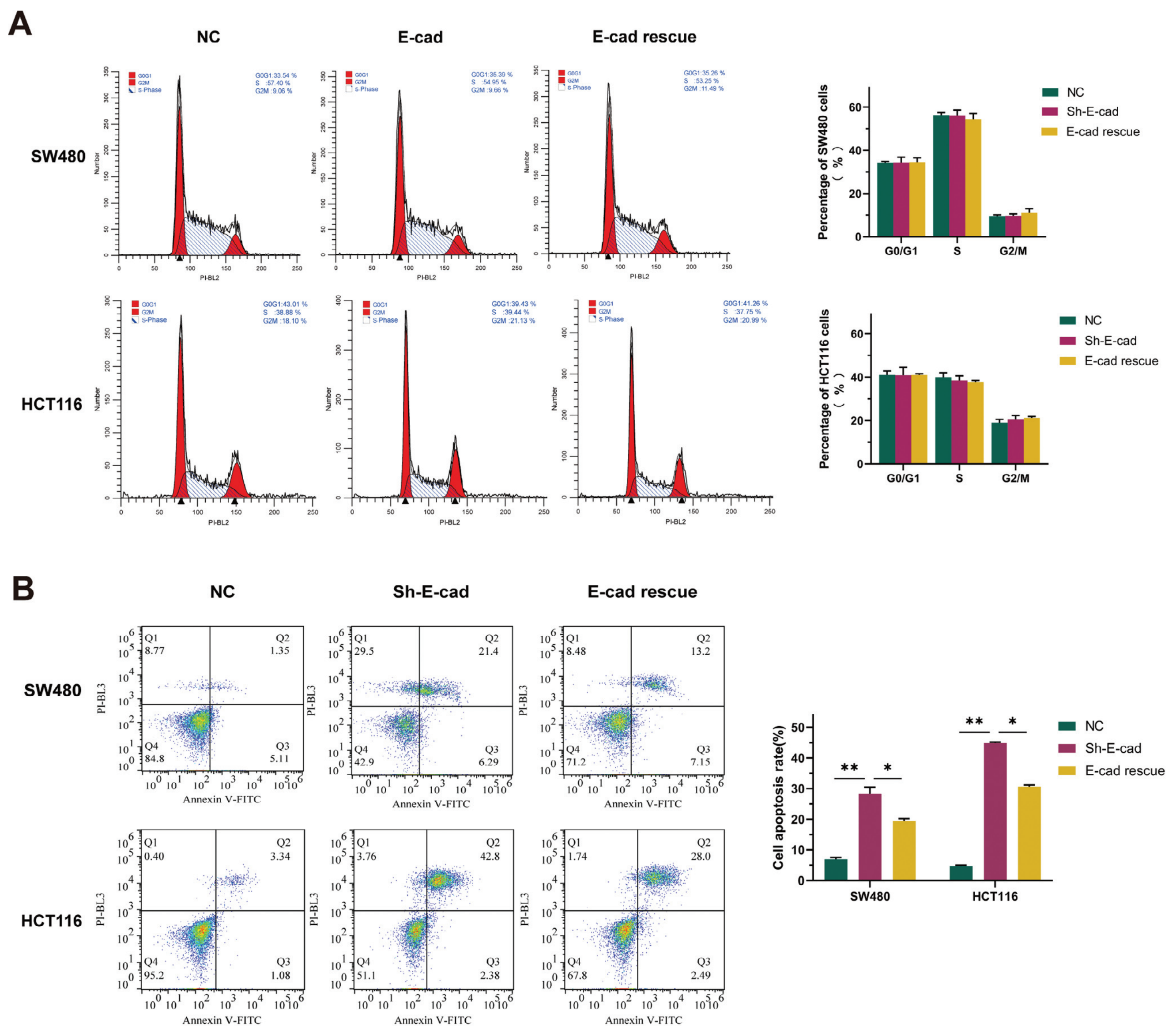

Figure 3. E-cad inhibits the apoptosis in CRC cells. **A**) Cell cycle analysis of HCT116 cells and SW480 cells. **B**) Apoptosis analysis of HCT116 cells and SW480 cells. Data are presented as mean ±SEM (n=6 per group). *p<0.05, **p<0.01.

existing chemotherapy or immunotherapy. E-cad is a crucial cell adhesion protein predominantly expressed in epithelial cells, responsible for mediating adhesion between cells and maintaining tissue structure integrity.[17] Research has shown that the expression levels and functionality of E-cad are closely associated with cancer development, metastasis, and prognosis. A decrease in E-cad expression can weaken cell adhesion, thereby facilitating the invasiveness and metastatic potential of tumor cells.[18] In colon cancer, the inactivation or downregulation of E-cad has often resulted in tumor progression and malignancy. During the metastatic process of CRC, reduced E-cad expression is highly correlated with EMT, a biological process that enhances the migratory and invasive abilities of tumor cells. Studies have indicated that PCSK9 directly or indirectly upregulates Snail 1, leading to the reduction of E-cad expression while simultaneously increasing the levels of N-cadherin and MMP9, thus inducing EMT in colon cells and activating the PI3K/AKT signaling pathway, which enhances colon cancer cell metastasis.[19] Additionally, the impact of E-cad gene mutations

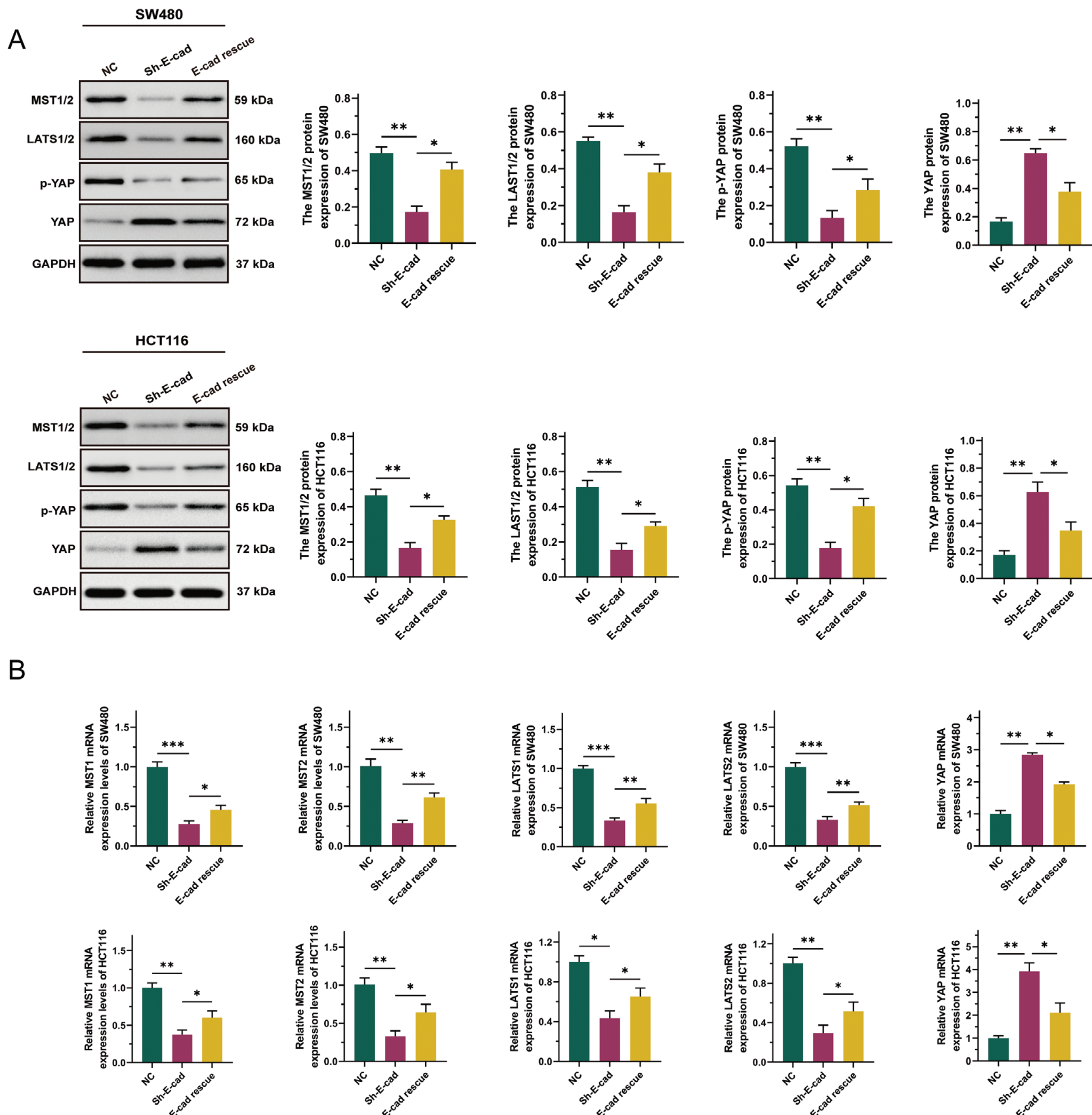

Figure 4. E-cad regulated the Hippo signaling pathway in CRC cells. **A**) The expressions of MST1/2, LATS1/2, p-YAP and YAP proteins in SW480 cells (upper raw) and in HCT116 cells (lower raw). **B**) The expressions of of MST1/2, LATS1/2, p-YAP and YAP mRNAs in SW480 cells (upper raw) and in HCT116 cells (lower raw). Data are presented as mean ±SEM (n=6 per group). *p<0.05, **p<0.01, ***p<0.001.

or methylation status on the prognosis of colon cancer patients has been documented.[20] Restoring E-cad expression or function may offer potential to inhibit tumor progression and metastasis. Research has shown that TGF-β-Smad2/3-E-cad signaling is a key pathway that mediates anti-tumor and anti-metastatic effects of *Patrinia villosa* aqueous extracts in colon cancer.[21] Furthermore, the study revealed that Genistein induces tumor cells apoptosis by reversing EMT through the Notch1/NF-κB/Slug/E-cad signaling pathway.[22] In osteosarcoma cells, E-cad was upregulated by (S)-10-hydroxycamptothecin dose-dependently, and this effect was mediated by Hippo signaling pathway and reversing EMT.[23] These findings, consistent with our results, demonstrates that silencing E-cad facilitates the clonogenicity, migration, and proliferation of CRC cells. Conversely, restoring E-cad expression partially attenuated these capabilities. These results conclude that E-cad-mediated EMT plays a critical part in the progression of CRC. Interestingly, through further investigation, we found that E-cad did not significantly alter the cell cycle of CRC cells but had a marked impact on their apoptosis. Numerous studies indicate that the Hippo pathway primarily regulates organ volume by inhibiting cell proliferation and inducing apoptosis. The kinase cascade is a critical component of this signaling pathway.[24] Mst1/2 kinases combines with SAV1 and form a complex, which subsequently phosphorylates LATS1/2. Then the activated LATS1/2 kinases phosphorylate YAP/TAZ and inhibiting their transcriptional activity. Unphosphorylated YAP/TAZ, on the contrary, can bind to TEAD1-4 or other transcription factors in the nucleus, leading to the upregulation of both the pro-proliferative and anti-apoptotic

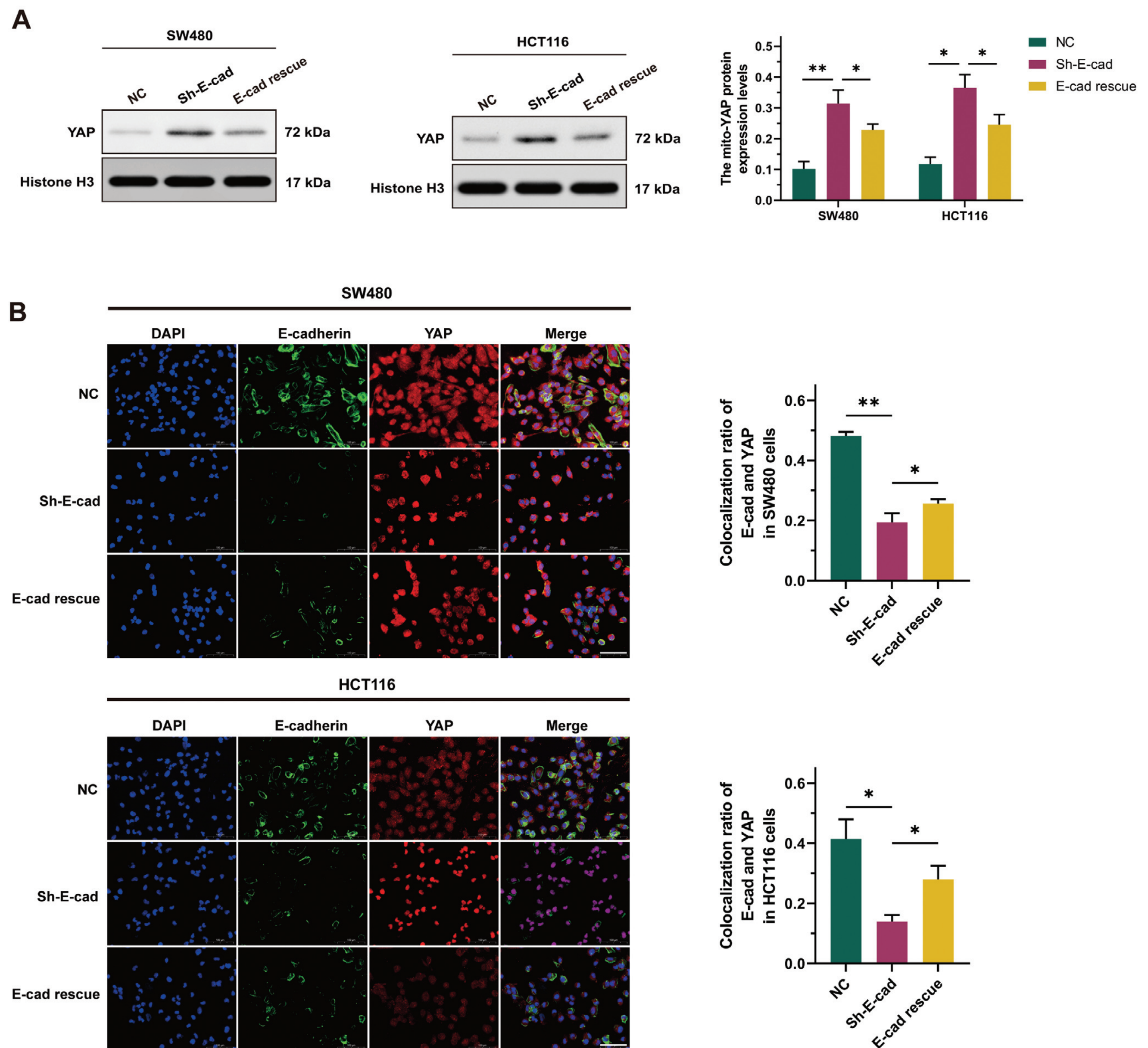

Figure 5. E-cad upregulated the nuclear translocation of YAP. **A**) The nuclear translocation of YAP in HCT116 cells. **B**) The co-localization of E-cad and YAP. Scale bar: 100 µm. Data are presented as mean ±SEM (lymph node metastasis). *p<0.05, **p<0.01.

gene expression.[25] The regulatory effect of the Hippo pathway on organ size has been reported in recent animal studies. For instance, specific over-expression of YAP in the liver contributes the increase of organ volume, however, once YAP over-expression is halted, liver size can return to normal.[26] The expression and function of E-cad can activate the Hippo signaling pathway.[27] When cell adhesion is robust, E-cad can promote the signaling transduction of the Hippo pathway by inhibiting YAP/TAZ activity, thereby helping to suppress cell proliferation and maintain epithelial characteristics.[28,29] Our experimental results also suggest that following the silencing of E-cad, the expression levels of MST1/2 and LATS1/2 significantly decreased, while unphosphorylated YAP levels increased, leading to enhanced cell proliferation. Silencing of E-cad resulted in a significant increase in YAP nuclear translocation, indicating that E-cad regulates CRC progression by regulating Hippo signaling pathway.

In this study, reduced phosphorylation level of YAP in CRC cells synchronously with E-cad silencing has a significant implication for the activity of YAP on the CRC progression. Typically, when phosphorylation levels of YAP are reduced due to E-cad silencing, unphosphorylated YAP accumulates in the nucleus.[30] As a transcriptional co-activator, YAP can promote the expression of genes associated with cell proliferation, survival, and resistance to apoptosis.[31] In CRC cells, the increased nuclear translocation of YAP enhances these processes, contributing to tumor angiogenesis by accelerating cell proliferation and reducing apoptosis.[32] Moreover, YAP's activation can drive EMT by interacting with extracellular matrix, which promotes cell migration, invasion and metastasis.[33] Therefore, therapies targeting downstream molecular of the Hippo pathway could be another potential strategy that inhibiting YAP-mediated oncogenic effects in CRC. Furthermore, understanding this mechanism may lead to the development of novel biomarkers for predicting CRC progression.

One limitation of this study is no further verification of CRC animal model was conducted. The complexity of tumor microenvironment which involved with cell-cell interaction, cell-matrix interaction, immune homeostasis is difficult to mimic entirely. Although we observe an intrinsic relationship between E-cad and YAP in CRC cells, the mechanism is worth to be explored and verified by a more well-designed *in vivo* experiment in the future. The current result definitely has built a foundation for our next endeavor in where a novel E-cad analogue or agonist will be applied to CRC mice model and its value in suppressing CRC development will be revealed. In addition, despite of regulation of E-cad on the protein level of YAP in nuclear is confirmed, the functional analysis of YAP's transcriptional activity by luciferase reporter assay should be also involved in the design to demonstrate the interaction dynamically.

In summary, E-cad suppresses the malignant progression of CRC through activating the Hippo signaling pathway. This provides a theoretical basis for developing targeted therapies against E-cad and offers potential strategy for the early diagnosis and prognosis estimation of CRC.

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