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Rapamycin Potentiates the Antitumor Effect of 5-Fluorouracil in Colon Cancer by Enhancing Autophagy

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Abstract: *Objective:* To investigate whether the mTOR inhibitor rapamycin can enhance the growth suppression effect of 5-fluorouracil (5-FU) on colon cancer cells. *Methods:* CCK8 assays were used to examine cell survival. Flow cytometry and Western blotting were employed to detect cell proliferation, apoptosis, and related markers. *Results:* The combination of rapamycin and 5-FU exhibited greater cytotoxicity in cells compared to rapamycin or 5-FU alone. Notably, cells in the G_0/G_1 phase increased while cells in the S phase decreased in the combination group, consistent with changes in the levels of Cyclin D1 and PCNA. Rapamycin enhanced 5-FU-induced apoptosis *in vitro* by inducing caspase-dependent apoptosis, which is Bax/Bcl-2 related. *Conclusion:* The combination of rapamycin and 5-FU showed a significant synergistic anticancer effect by enhancing autophagy. This study supports that the combination of rapamycin with 5-FU is an effective and feasible approach for colorectal cancer treatment.

Keywords: Colorectal cancer; Proliferation; Apoptosis; Autophagy; Rapamycin

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1. Introduction

Colorectal cancer (CRC), characterized by uncontrolled cell growth, ranks third among gastrointestinal tumors, posing a significant health threat. Its treatment involves surgery, chemotherapy, radiotherapy, immunotherapy, or a combination thereof [1]. 5-Fluorouracil (5-FU), a prevalent colon cancer chemotherapy drug, inhibits thymidylate synthase, halting thymine production to stop cell growth and DNA replication, thereby triggering apoptosis [2]. However, chemotherapy resistance and adverse effects limit the efficacy of 5-FU [3].

Mammalian target of rapamycin (mTOR) inhibitors may play a role in overcoming drug resistance [4]. mTOR is an important signaling pathway involved in various cell processes and is highly activated in CRC. In contrast, inhibiting the PI3K-Akt-mTOR pathway can effectively suppress the growth of CRC cells both *in vitro* and *in vivo* [5,6]. The mTORC1 inhibitor rapamycin, known for its anti-proliferative and anti-tumor effects, has been approved by the U.S. Food and Drug Administration for the treatment of renal cell carcinoma.

Combination drug therapy may overcome the current limitations of cancer therapy. To date, little is known about whether the combination of 5-FU with rapamycin can maximize efficacy. This study found that 5-FU combined with rapamycin caused synergistic growth arrest and cooperative pro-apoptotic effects in CRC.

Autophagy plays a crucial and paradoxical role in determining the fate of cells, acting as both a death inducer and death effector in response to various anticancer treatments [7,8]. The anticancer effect of 5-fluorouracil in colorectal cancer can be promoted by autophagy [9]. Rapamycin is an autophagy activator, so it is hypothesized that rapamycin enhances the activation of autophagy-mediated cell death in colon cancer.

2. Materials and methods

2.1. Cell lines

Human HCT116 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM containing 10% fetal bovine serum (FBS) at 37°C with a CO_2 concentration of 5%.

2.2. Cell viability

Cell viability was measured using the Cell Counting Kit-8 (CCK-8, DojinDo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, a 96-well plate was inoculated with cells at a density of 1×10^4 cells/well, with each group having three replicates. CCK-8 reagent (10 μL) was added to each well and incubated for 1 hour. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, USA). Three separate experiments were conducted.

2.3. Proliferation assays

Cell proliferation was measured with the eBioscience™ BrdU Staining Buffer Set for Flow Cytometry (Invitrogen™). After the final washing step, cells were resuspended in PBS containing 0.5% BSA and 7-aminoactinomycin D (Via-PROBE, Becton Dickinson) for cell cycle analysis [10].

2.4. Apoptotic assays

Apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (4ABio, China) according to the manufacturer's instructions.

2.5. Western blotting

Western blotting was performed using an SDS-PAGE electrophoresis system. Whole-cell lysates were prepared using RIPA lysis buffer containing protease inhibitors, phosphatase inhibitors, and PMSF. Proteins were separated by a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with TBST (Tris-buffered saline containing 0.05% Tween-20) and 5% skim milk at room temperature. They were then incubated with primary antibodies against Cyclin D1, PCNA, Bcl-xl, Bcl-2, Bax, caspase-3, caspase-7, caspase-9, cleaved-caspase-3, cleaved-caspase-7, cleaved-caspase-9, and β-actin (Cell Signaling Technology) overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibodies at room temperature for 1 hour, washed three times, and observed with enhanced chemiluminescence (ECL) reagent (Thermo Scientific, USA). ImageJ software was used to analyze the band signal.

2.6. Statistical analysis

Statistical analysis was performed using Student's *t*-test with Prism software (GraphPad 7). Results are shown

as mean ± standard deviation (SD) in triplicate. ANOVA and Tukey's multiple comparisons were appropriately used. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. The combination of rapamycin and 5-FU possesses synergistic cytotoxic effects on colorectal cancer cells

CCK-8 assay showed that 5-FU alone inhibited cell growth in a concentration- and time-dependent manner, while rapamycin alone had a weak inhibitory effect on cell growth, with no further inhibitory effect after increasing the concentration of rapamycin (**Figure 1A** and **B**). However, the combination of rapamycin with 5-FU increased the inhibition rate of cell growth in a time- and concentration-dependent manner (**Figure 1B**). The synergistic index (CI) was less than 1, indicating a better cytotoxic activity calculated by ComboSyn software, showing that the combination of 5-FU and rapamycin had a significant synergistic effect on colorectal cancer cells (**Figure 1C**).

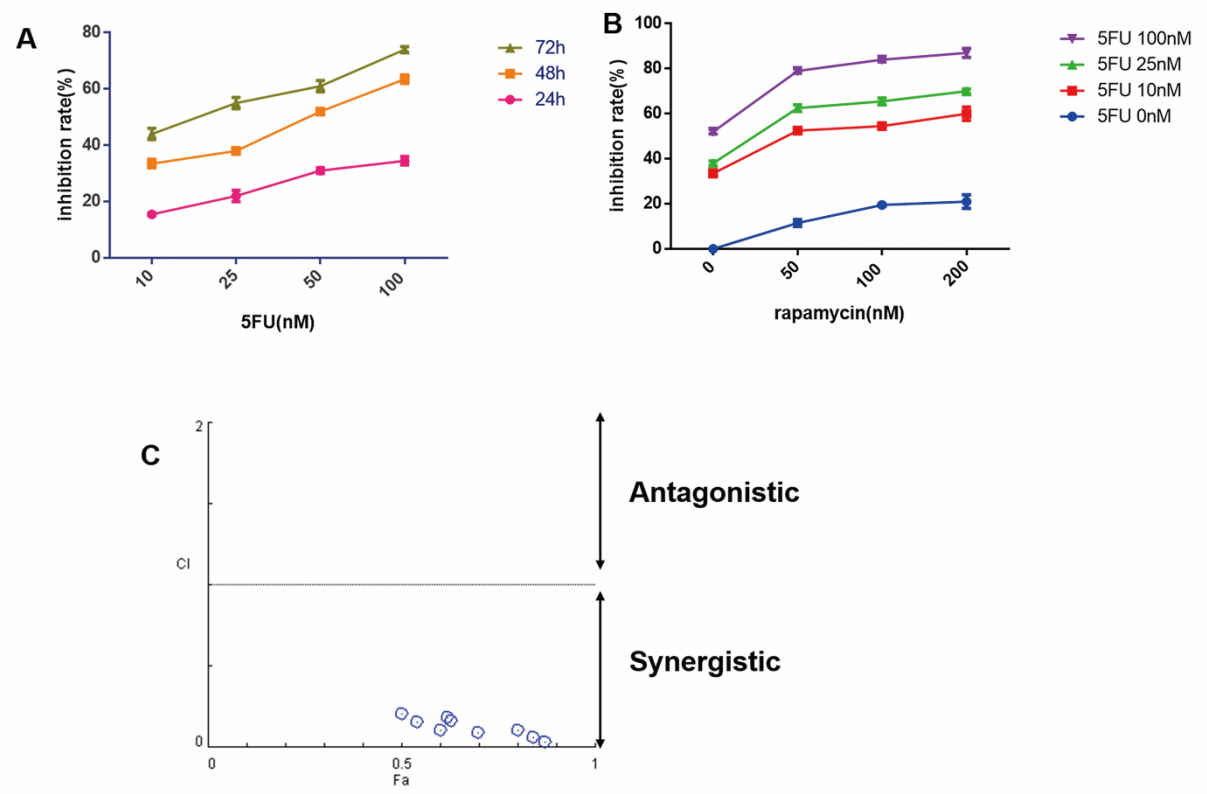

Figure 1. Rapamycin synergistically enhances the cytotoxic effect of 5-FU. **(A)** Cell inhibition rate measured by CCK-8 assay when HCT-116 cells were treated with 5-FU (10, 25, 50, and 100 nM) alone for 24h, 48h, and 96h; **(B)** Cell inhibition rate determined when HCT-116 cells were treated with rapamycin and 5-FU alone or in combination for 48h; **(C)** CI value calculated by ComboSyn software after treatment with a combination of rapamycin and 5-FU for 48h

3.2. 5FU combined with rapamycin causes synergistic proliferation arrest of colorectal cancer cells

Abnormal proliferation is a key characteristic of tumor cells, typically assessed by counting S-phase cells. BrdU assay efficiently measures S phase cell numbers. In this study, flow cytometry and statistical data showed that the proportion of cells in the S phase (cells mixed with BrdU in DNA molecule) in the combination group was

significantly lower than that in the single group, indicating that 5-FU combined with rapamycin can suppress cell proliferation (**Figure 2A** and **B**).

Cell proliferation and differentiation are closely related to the cell cycle, and uncontrolled cycle regulation may lead to unlimited cell proliferation, stimulate cell division, and further destabilize chromosomes. The cell cycle was detected by double-labeling with 7-ADD and BrdU antibody binding dye (**Figure 2A** and **B**). The results showed that compared with the control group, 5-FU combined with rapamycin induced a significant increase in G_1/G_0 cells. Notably, this increase paralleled a significant simultaneous decrease in S-phase cells, resulting in cell cycle arrest and subsequent inhibition of cell growth.

Cyclin D1 and PCNA, key factors in cell cycle progression, are crucial for tumor genesis and growth. Western blot analysis revealed that 5-FU and rapamycin, either alone or combined, significantly reduced Cyclin D1 and PCNA protein expression compared to the control. The combination of rapamycin with other drugs showed a more pronounced decrease in Cyclin D1 and PCNA expression (**Figure 2C** and **D**).

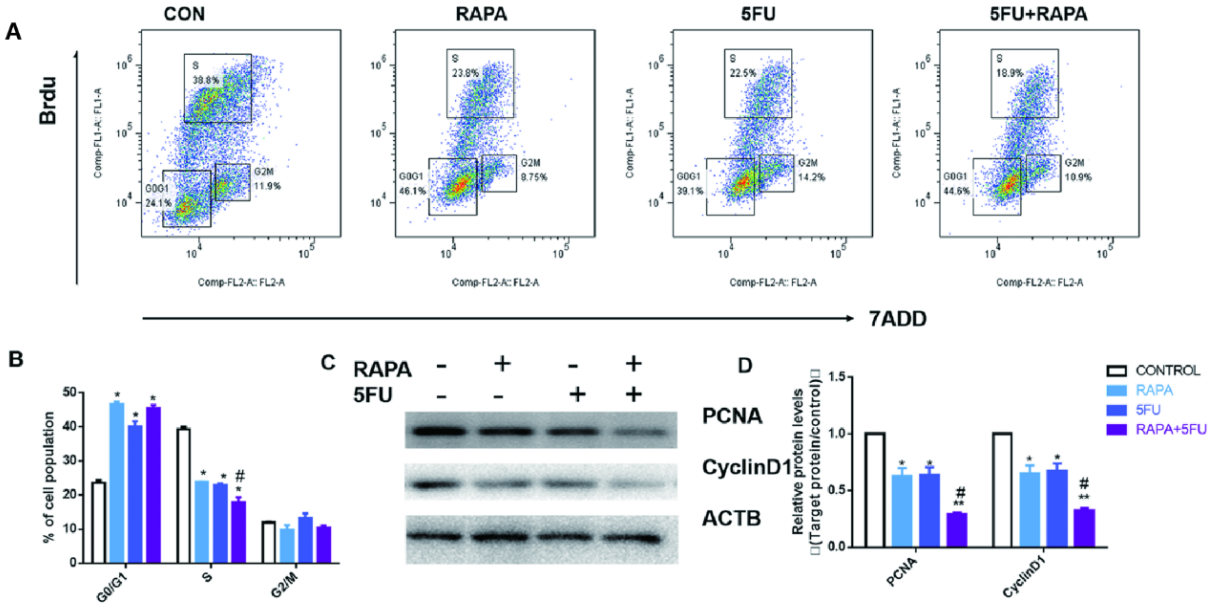

Figure 2. 5-FU combined with rapamycin inhibited the proliferation of HCT-116 cells. **(A)** Representative photomicrographs of the cell cycle distribution in different groups for 48h (Control, rapamycin, 5-FU, rapamycin combined with 5-FU); **(B)** The flow cytometry assay for cell cycle distribution; **(C)** Expression of PCNA and Cyclin D1 proteins in HCT-116 cells; **(D)** The quantitative analysis of the protein expression normalized to ACTB. **P* < 0.05 vs. control, ***P* < 0.01 vs. control; # *P* < 0.05 vs. 5-FU, ## *P* < 0.05 vs. 5-FU

3.3. 5FU combined with rapamycin synergistically induces apoptosis of colorectal cancer cells

Programmed cell death (PCD) is an intracellular programmed regulation of cell death, with apoptosis characterized by nuclear and chromosomal DNA fragmentation in type I PCD cells. Abnormal apoptosis, along with malignant hyperplasia, contributes to tumor pathogenesis. 5-FU induced significant apoptosis, while rapamycin alone had minimal effect. However, the combined administration of 5-FU and rapamycin had a more significant effect on inducing apoptosis (**Figure 3A**).

These results were further confirmed by evaluating the expression levels of apoptosis-related proteins. The increased BAX/Bcl-2 ratio, indicating higher pro-apoptotic BAX and lower anti-apoptotic Bcl-2 family

proteins, suggests apoptosis induction by promoting mitochondrial membrane permeability disruption [11]. Down-regulation of the anti-apoptotic BCL-2 protein family activates caspase-9, which further activates caspase-3 and caspase-7, promoting an inherent apoptotic process of caspase cascade amplification [12-14]. According to Western blot analysis, 5-FU alone increased cleaved caspase-3, caspase-7, caspase-9, and BAX expression levels, while decreasing BCL-2 and BCL-XL (**Figure 3B**). Rapamycin alone modestly decreased BAX-xl and BCL-2, but in combination with 5-FU, it markedly enhanced apoptosis by reducing BCL-2 family interactions with BAX. This led to lower BCL-2 and BCL-XL levels, activating caspase-3, -7, and -9. Flow cytometry data support that the 5-FU + rapamycin combo partially induces colorectal cancer cell apoptosis via mitochondrial and caspase-dependent pathways.

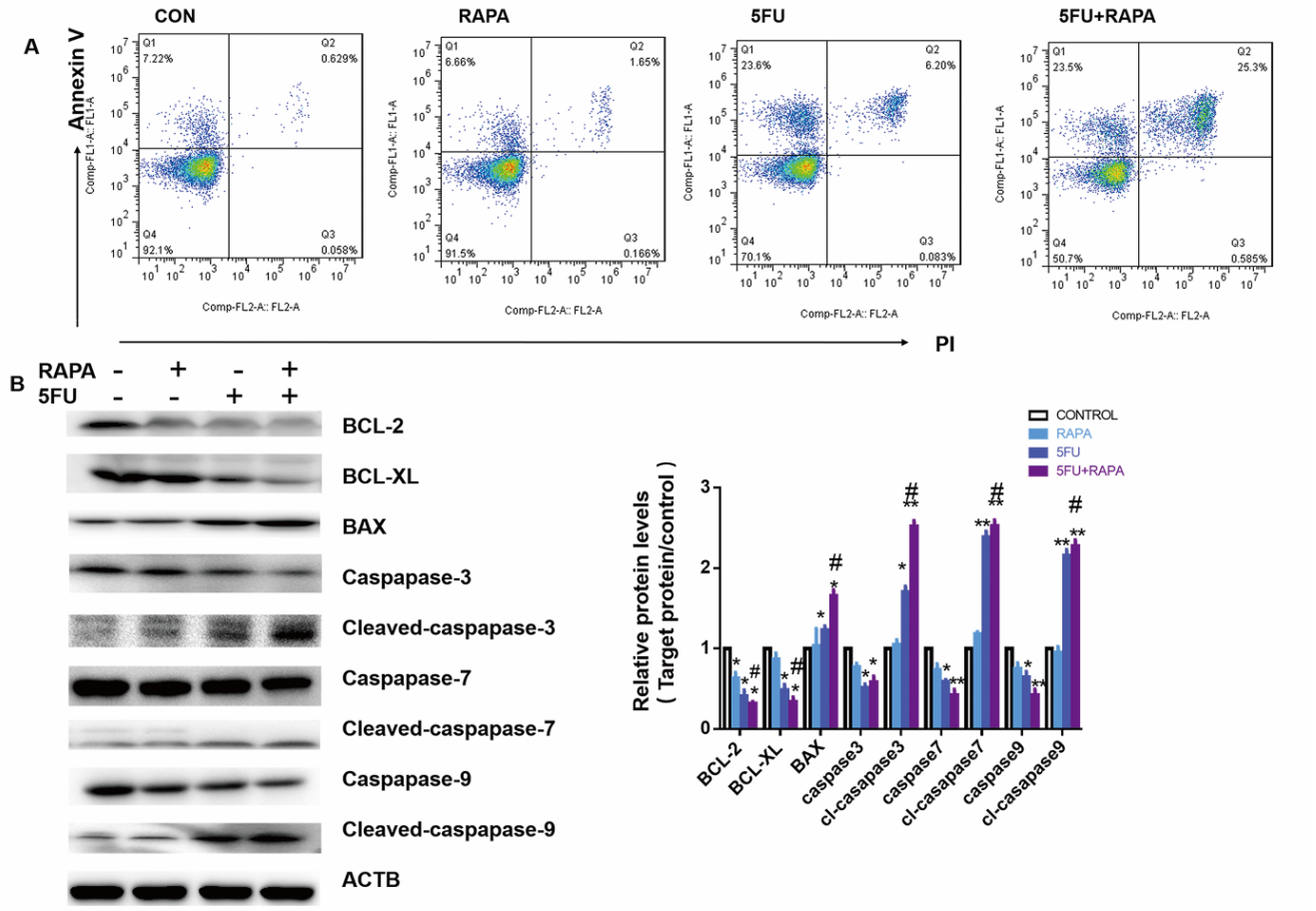

Figure 3. Rapamycin enhances the pro-apoptotic effect of 5-FU. **(A)** The percentage of apoptotic cells analyzed by flow cytometry; **(B)** The expression levels of apoptosis-related proteins analyzed by Western blot. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control; # $P < 0.05$ vs. 5-FU, ## $P < 0.05$ vs. 5-FU

3.4. 5-FU combined with rapamycin induces autophagy in colorectal cancer cells

Autophagy, a type of cell death lacking apoptotic features, is associated with solid tumor formation and regulates cell survival and death processes. Abnormal autophagy is linked to various diseases, including cancer [15]. Beclin-1, a positive regulator of autophagy and a tumor suppressor, and LC3-II, a major marker of autophagy-related processes, were considered markers of autophagy. This study examined the expression of autophagy-related proteins (**Figure 4**). Compared with the control group, the levels of Beclin-1, LC3-II, and p62

in the 5-FU group did not change significantly, indicating that 5-FU did not induce autophagy in colon cancer cells. Rapamycin increased Beclin-1 and LC3-II levels and decreased p62 levels, suggesting that rapamycin induces autophagy in colon cancer cells. The combination of rapamycin and 5-FU further exaggerated this effect. In summary, these results confirm that the combination of enhanced autophagy and apoptosis in colon cancer cells can regulate autophagy-induced apoptosis.

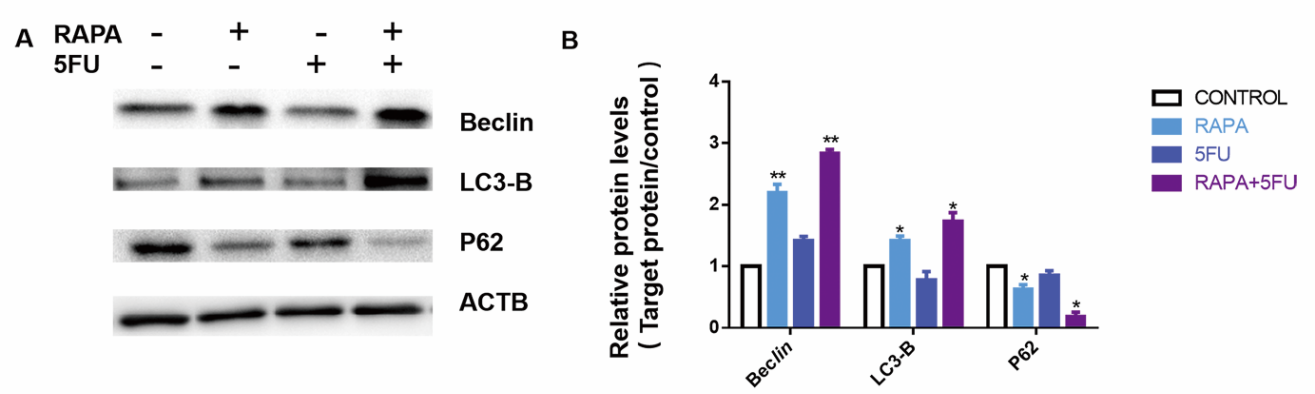

Figure 4. Autophagy induced by co-treatment with rapamycin and 5-FU in HCT-116 cells. The expression levels of autophagic proteins (LC3-II and Beclin-1) were analyzed by Western blot. **P* < 0.05 vs. control, ***P* < 0.01 vs. control; # *P* < 0.05 vs. 5-FU, ## *P* < 0.05 vs. 5-FU.

4. Discussion

5-FU is the most common chemotherapy agent for colorectal cancer [9]. The combination of PI3K/mTOR inhibitors and HDAC inhibitors shows promise in treating colorectal cancer, overcoming 5-FU resistance, and minimizing side effects, offering a more effective and tolerable chemotherapy approach compared to single-drug treatments [16-18]. It has been reported that drug resistance to 5-FU may be mediated by the mTOR pathway [19]. mTOR inhibitors have been reported to overcome 5-FU resistance, making 5-FU-resistant cell lines sensitive and inhibiting the growth of gastric cancer cells [20]. Rapamycin can bind to the FKBP12-rapamycin domain of mTOR, inhibiting mTOR activity. Shigematsu *et al*. reported that rapamycin enhanced the cytotoxic effect of 5-FU in gastric cancer cells [21]. Combined treatment with 5-FU and rapamycin can synergistically inhibit the growth of colon cancer cells [9]. This study's results also suggest that rapamycin may increase the sensitivity of cancer cells to 5-FU, contributing to the improvement of clinical efficacy.

Cell proliferation and apoptosis balance maintain tissue homeostasis, and disruptions can lead to diseases, including tumors, resulting from uncontrolled cell growth or apoptosis resistance [22]. This study shows that combining rapamycin with 5-FU enhances the therapy's impact on colon cancer cells by inhibiting proliferation, promoting cell cycle arrest, and increasing pro-apoptotic markers while reducing anti-apoptotic proteins, leading to more effective apoptosis than either drug alone.

Autophagy may be associated with the development and progression of CRC. Beclin-1 is a positive autophagic regulator and a tumor suppressor [23], while LC3-II is considered a reliable marker involved in monitoring autophagy and its related processes [24]. Overexpression of Beclin-1 and LC3 has been reported to predict favorable survival in various cancers [25]. The combination of the PI3K/mTOR inhibitor BEZ235 and the histone deacetylase inhibitor TSA synergistically inhibited esophageal cancer cell activity and induced autophagy, enhancing killing effects and proliferation inhibition, while upregulating Beclin-1 and LC3-II expression [26]. After inhibiting autophagy by targeting the AKT/rapamycin signaling pathway, drug-resistant

cells become sensitive to 5-FU-induced apoptosis [27]. Beclin-1 overexpression promotes proliferation inhibition, while Beclin-1 down-regulation reduces the autophagy of 5-FU and weakens its anti-tumor effect [9]. This study found that rapamycin increased LC3-II and Beclin-1 expression, amplifying 5-FU's apoptotic effect on HCT116 cells by activating autophagy, which may lead to organelle and protein self-digestion and apoptosis.

5. Conclusion

In conclusion, rapamycin combined with 5-FU inhibits the survival of colon cancer cells by inducing apoptosis and autophagy *in vitro*, which is an effective and feasible treatment for colorectal cancer.

Authors' contributions

Conceptualization: Xiaoming Tian, Youjun He, Tao Hao
Investigation: Xiaoming Tian
Formal analysis: Xiaoming Tian, Youjun He, Daqi Huang, Lei Li
Writing – original draft: Xiaoming Tian, Tao Hao
Writing – review & editing: Xiaoming Tian, Youjun He, Daqi Huang, Lei Li, Tao Hao

Disclosure statement

The authors declare no conflict of interest.

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