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Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer *in vitro* and *in vivo* model

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ABSTRACT

Although 5-fluorouracil (5-FU)-based adjuvant chemotherapy is widely used in the treatment of colorectal cancer, novel therapeutic strategies need to be explored. It has been reported that autophagy is extensively implicated in cancer. However, the function of autophagy is not fully understood. In the present study, apoptosis induced by 5-FU in 3 human colon cancer cell lines (HCT116, DLD-1, and DLD-1/5-FU (a specific 5-FU-resistant sub-line)) was measured using MTT assay, DNA fragmentation assay, Hoechst 33342 staining, and caspase-3 immunoblotting. The autophagy activation induced by 5-FU treatment was revealed by microtubule-associated protein 1 light chain 3 (LC3) immunofluorescence and immunoblotting and p62 immunoblotting. Inhibition of autophagy by 3-methyladenine (3-MA) or small interference RNA targeting Atg7 (Atg7 siRNA) significantly augmented 5-FU-induced apoptosis. This synergistic effect of 5-FU and 3-MA was further confirmed in the DLD-1 xenograft tumour model. Tumour growth was suppressed more significantly with combination treatment than 5-FU treatment alone. In conclusion, autophagy was activated as a protective mechanism against 5-FU–induced apoptosis and its inhibition could be a promising strategy for adjuvant chemotherapy in colon cancer.

1. Introduction

Colorectal cancer (CRC) is the second most prevalent cancer and the third leading cause of cancer deaths worldwide. Surgery is the primary treatment for CRC. However, most patients with metastasis are candidates for systemic chemotherapy to palliate symptoms and prolong life.[1,2] At the present time, 5-fluorouracil (5-FU) remains the cornerstone of systemic treatment for colorectal cancer. 5-FU has been widely used for stage III colon cancer and stage III rectal cancer since the 1980s. In the 1990s, the 5-FU/leucovorin protocol was accepted as a standard for CRC treatment.[3,4] However, along with its usage, resistance to 5-FU has become common and has been recognised as a reason for CRC therapy failure. Although many aggressive adjuvant therapies such as irinotecan or oxaliplatin combined with 5-FU are being tested in clinical treatment, a novel chemotherapy combination still needs to be explored.[5]

Autophagy is an evolutionary conserved eukaryotic process in which organelles and bulk proteins are turned over by lysosomal activity. The most distinctive feature of autophagy is the formation of autophagosomes, double- membrane vesicles that fuse with lysosomes for hydrolytic cleavage of engulfed proteins and organelles.[6] In mammalian cells,

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microtubule-associated protein 1 light chain 3 (LC3) is conjugated to phosphatidyl ethanolamine for insertion into the autophagosome membrane. LC3-II (a lipidated form of LC3) has been deemed a marker of autophagy and detecting LC3-II by immunoblotting or immunofluorescence is a reliable method for monitoring autophagosome formation.[7,8] Moreover, p62 protein has recently been reported as an autophagy substrate whose level decreases upon autophagy induction; as such, it has been used to monitor autophagy flux.[8,9]

The physiologic function of autophagy is to maintain homeostasis by eliminating unnecessary proteins and injured or aged organelles. Recently, increasing evidence has shown that autophagy is also associated with many pathologic conditions such as neurodegenerative diseases, hereditary myopathies, and cancer.[10] Many studies have focused on the relationship between autophagy and tumour pathogenesis, development, and treatment. But autophagy seems to play a paradoxical role in cancer cell survival and death.[11–13] Moreover, chemotherapy also induces autophagy in cancer. Because studies about autophagy improving survival or inducing death both exist,[14–17] whether autophagy enables cells to survive or enhances their death is context-driven and depend on the stimuli type, nutrient availability, organism development, and apoptotic status.[18,19]

In our previous study, we found that the autophagy inhibitor 3-MA enhanced the effect of 5-FU-induced apoptosis in 2 colon cancer cell lines, HT29 and colon26.[20] This suggested that targeting autophagy inhibition could be a promising strategy during 5-FU chemotherapy in colon cancer. However, since colon cancer is heterogeneous,[21] the application is limited. So, in this study, three other kinds of human colon cancer cells were used. To more directly detect the role of autophagy, Atg7 small interfering RNA (siRNA) were also applied and the resulting change of 5-FU effects was observed. Furthermore, DLD-1 xenografts in nude mice were used to confirm the role of autophagy *in vivo*.

2. Materials and methods

2.1. Cell culture

The human colorectal carcinoma HCT116 and DLD-1 cell lines were obtained from American Type Culture Collection. The 5-FU-resistant sub-line of the DLD-1 cell line (DLD-1/5-FU) was established by repeated, continuous exposure to 5-FU with stepwise escalation as described previously.[22] All cell lines were cultured with RPMI-1640 (Wako, Japan) supplemented with 10% foetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with medium changes every 2 d. Cells in a mid-log phase were used in this study.

2.2. Chemical reagents and antibody

5-FU and 3-MA were purchased from Sigma. Anti caspase-3, Bcl-2, Bcl-xl, Bad, Bax, p-p53, AMPK, p-AMPK, mTOR, p-mTOR, and p-p70S6k antibodies were purchased from Cell Signaling Technologies. Anti-Atg7 and -p53 were obtained from Santa Cruz Biotechnology. Anti-LC3 antibody was purchased from MBL and anti-p62 and anti-β-actin antibodies were obtained from Sigma.

2.3. RNA interference

Atg7 RNA interference was accomplished by transfecting HCT116, DLD-1, and DLD-1/5-FU cells with the specific siRNA. The Atg7-targeting sense sequence (5′-CCAACACACUCGAGUCUUU-3′) and the Universal Control siRNA were purchased from Invitrogen. Short oligo-RNAs were transfected using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Cells were cultured for 24 h before the experiment.

2.4. Measurement of cell viability and apoptosis

Cell viability was determined via MTT assay as described previously.[20] Cells were seeded in 96-well flat bottom microtitre plates at a density of 1×10^4 cells, 100 μl per well. After treatment as shown in the figure legends, 10 μl of the cell counting solution (WST-8, Dojindo laboratories, Tokyo, Japan) was added to each well and incubated in a humidified 5% CO_2 atmosphere at 37 °C for 3 h. Crystals were dissolved in 100 μl of 1 N HCl/well. The absorbance of the solution was read spectrophotometrically at 450 nm with a reference at 650 nm using a microtitre plate reader (Becton Dickinson). Cell viability was calculated according to the following formula: Cell viability (%) = A450/A450 (control group) × 100.

For the apoptosis assay, Hoechst 33342 staining and DNA fragmentation were used as described previously.[20] Cells were stained with Hoechst 33342 and examined under fluorescent microscopy (BX-50; Olympus). The apoptotic index (AI) was calculated as: [apoptotic cell number/total cell number] × 100 (%). At least four different fields from each well were selected; about 500 cells were counted to calculate the rate of apoptosis. Soluble DNA was extracted from both floating and attached cells after each treatment. Ten micrograms of DNA was separated with 2% agarose gel and visualised using UV transilluminator and photographed with Polaroid film.

2.5. Western blot analysis

Western blot analysis was performed as described previously.[20] Cells were extracted with lysis buffer [20 mM Tris–HCl, pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% NP-40, 1% aprotinin, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium vanadate]. Cell lysates (40 μg protein/line) were separated on a 5–20% Tris-Tricine Ready Gel SDS–PAGE (Bio-Rad) for nitrocellulose membrane blotting. The blotted membranes were blocked with 5% skim milk for 1 h and then incubated with primary antibodies (dilution 1:1000). The immunoreactive bands were visualised by enhanced chemiluminescence using horseradish peroxidase-conjugated IgG secondary antibodies. Band intensity was measured by densitometry, quantified using Gel Plotting Macros of NIH image 1.62 and normalised by an indicated sample in the same membrane.

2.6. Immunofluorescence for LC3

Immunofluorescence for LC3 was performed as described previously.[23] Cells on the chamber slide were washed with

PBS and fixed with 2% paraformaldehyde at 4 °C overnight. After fixation, the cells were permeabilized with 0.1% Triton X-100 for 15 min at room temperature and blocked with PBS containing 0.5% BSA and 0.5% glycine (BSA buffer) for 1 h at room temperature. Cells were then incubated with anti-LC3 (1:200 diluted in BSA buffer) antibody for 1 h at room temperature. After being washed with BSA buffer, cells were incubated with Alexa Fluor 488 conjugated anti-rabbit antibody (1:1000 diluted in BSA buffer) for 1 h at room temperature. Slides were mounted and examined using a fluorescence microscope (BX-50; Olympus).

2.7. *Xenograft tumour model in nude mice*

Female nude mice (BALB/c, nu/nu) were purchased from Japan SLC Inc., maintained under specific pathogen-free condi-

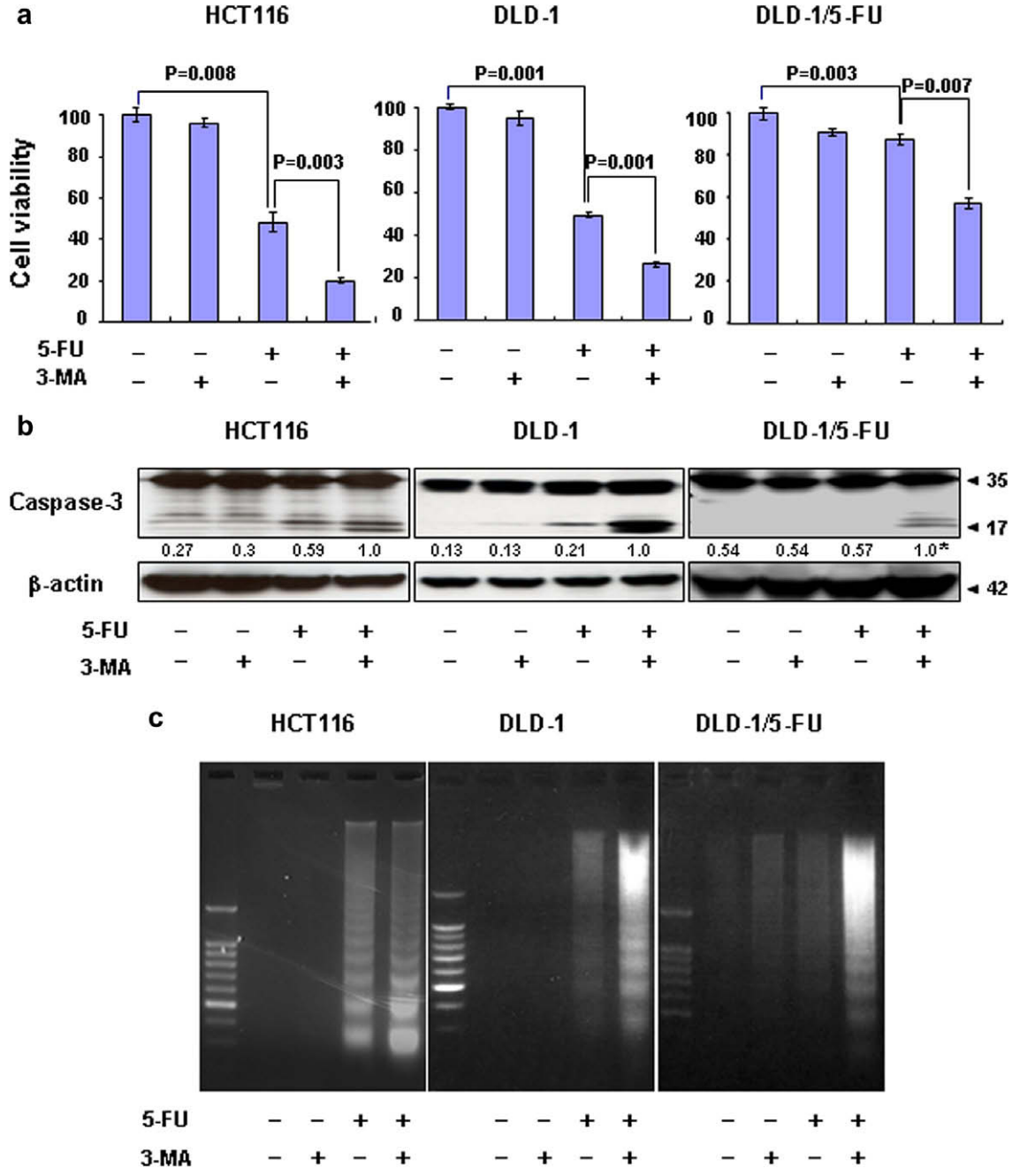

Fig. 1 – 3-MA enhances 5-FU-induced colon cancer cell death and apoptosis. HCT116, DLD-1 and DLD-1/5-FU cells were divided into four groups: control; 3-MA (5 mM); 5-FU (7 μM in HCT116, 50 μM in DLD-1, and DLD-1/5-FU); and combination (treated with 5 mM of 3-MA and the individual concentration of 5-FU) for 48 h. (a) Cell viability was measured via MTT assay. (b) Cell lysates were prepared and subjected to immunoblotting with antibodies to caspase-3 and β-actin. The values of the band intensity below the figure represent the densitometric estimation of each band normalised by β-actin. *The active form value. (c) DNA fragmentation assays were performed to observe DNA ladder formation. Data represent means of three independent experiments.

tions, and acclimated before the experiment. DLD-1 cells (5×10^6 cells) were injected subcutaneously in the right flank of 5-week-old mice (17–20 g in weight). Tumours were measured with calipers and tumour size was calculated using the formula: $V = 1/2\ ab^2$, where a represents the largest tumour diameter and b the smallest tumour diameter. The experiment was performed with the permission of the Animal Ethics Committee of Gunma University (Permission No. 08-079).

2.8. *In vivo combination treatment*

DLD-1 cells were injected in the right flank of nude mice. When the tumour size reached a predetermined size (180–350 mm^3, about 10 d after the injection), mice were randomly divided into control, 3-MA, 5-FU, and combination groups ($n = 5$ for each group). Thirty milligrams per kilogram of 5-FU and/or 24 mg/kg 3-MA dissolved in 100 μl saline were injected intraperitoneally every 5 d a total of 5 times (day 10, 15, 20, 25, and 30). As control, 100 μL of saline was injected as the control. Tumours were measured every 5 d and tumour volumes were calculated using the formula $V = 1/2\ ab^2$. After 1 month, tumour xenografts were excised and weighed. Tumour tissue protein extraction was then made and subjected to immunoblotting.

2.9. *Statistical analysis*

Statistical analysis between the two groups was calculated using Student's *t*-test, and multiple groups were performed by SPSS program version 10.1 (San Rafael, CA). Differences were considered significant when the *P* value was less than 0.05.

3. Results

3.1. *5-FU-induced colon cancer cell death was enhanced by 3-MA treatment*

Three kinds of human colon cancer cells, HCT116, DLD-1, and DLD-1/5-FU (a 5-FU–resistant sub-line of DLD-1), were used and the IC_{50} for 48-h 5-FU treatment was examined via MTT assay. The IC_{50} in HCT116, DLD-1, and DLD-1/5-FU was 7.09 ± 0.87 μM, 47.25 ± 5.97 μM, and 2149 ± 160 μM, respectively (Supplementary Fig. S1). As a result, we used 7 μM of 5-FU in HCT116 and 50 μM in DLD-1 and DLD-1/5-FU for 48 h in the subsequent experiment.

Next, changes in colon cancer cell death were examined using 5-FU with or without 3-MA. By 5-FU treatment, viability of HCT116 and DLD-1 decreased to 48.38% and 49.67%. With addition of 3-MA, cell deaths were enhanced to 79.9% and 73.76%, respectively. Although cell death in DLD-1/5-FU by 50 μM of 5-FU treatment was limited (12.34%), use of both 3-MA and 5-FU increased cell death to 42.72%, about 2.5 times higher than with use of 5-FU alone (Fig. 1a). There was no significant cell death by treatment of 3-MA alone in these cells. Next, apoptosis during the experiment was examined by immunoblotting of caspase-3 (Fig. 1b). In both 5-FU-treated HCT116 and DLD-1 cells, caspase-3 was cleaved into its active forms (from 35 kDa to 19 and 17 kDa). Further addition of 3-MA made the activated forms increase about 69.5% in HCT116 and 3.76 times in DLD-1 cells compared with treatment of 5-FU alone. Use of 50 μM of 5-FU could not activate caspase-3 in DLD-1/5-FU cells, although addition of 3-MA caused remarkable activation. The DNA fragmentation assay was further used to detect DNA ladder formation, the characteristic sign of apoptosis (Fig. 1c). The 5-FU-treated HCT116 and DLD-1 cells showed characteristic DNA ladder formation, which addition of 3-MA made more prominent. The DNA ladder in the 5-FU–treated DLD-1/5-FU cells was barely percepti-

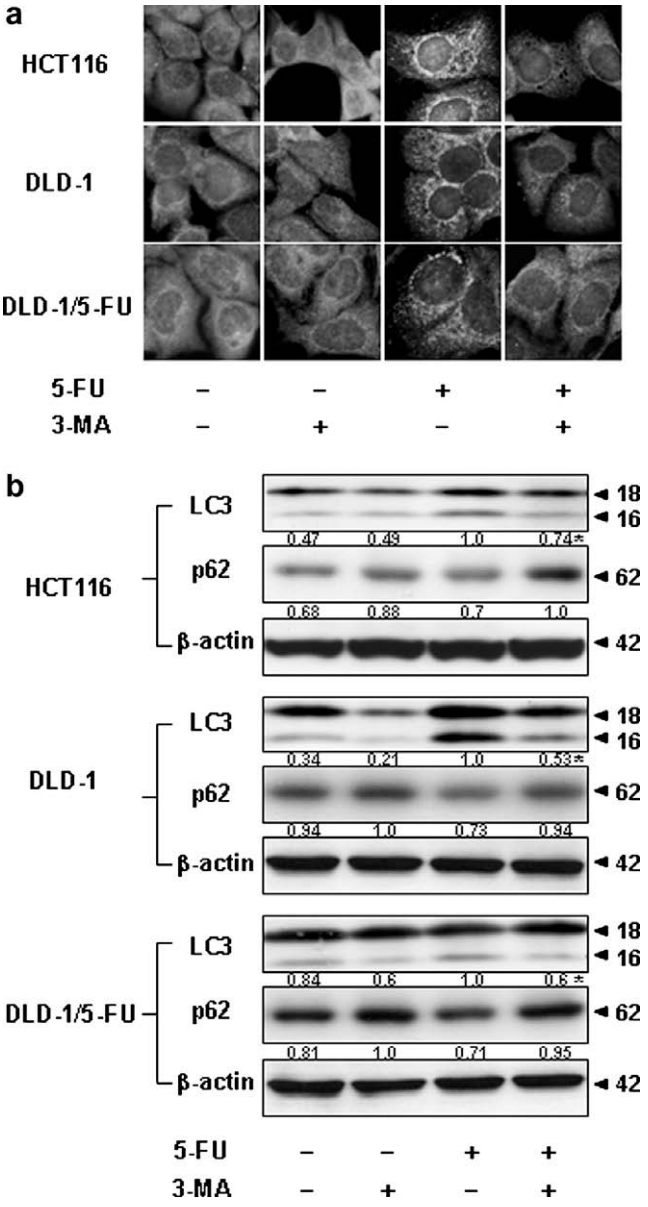

Fig. 2 – 3-MA inhibits the increased autophagy induced by 5-FU. HCT116, DLD-1, and DLD-1/5-FU cells were treated as in Fig. 1. (a) LC3 staining was performed to observe autophagosome formation. (b) Immunoblotting for LC3 and p62 were used to observe autophagy. The values of the band intensity below the figure represent the densitometric estimation of each band normalised by β-actin. *The LC3-II value. Photos (×400) and values are representatives of three independent experiments.

ble, but it appeared when 3-MA was simultaneously added. These results suggest that 3-MA enhanced 5-FU-induced cell death of these colon cancer cells by enhancing the caspase-mediated apoptosis pathway.

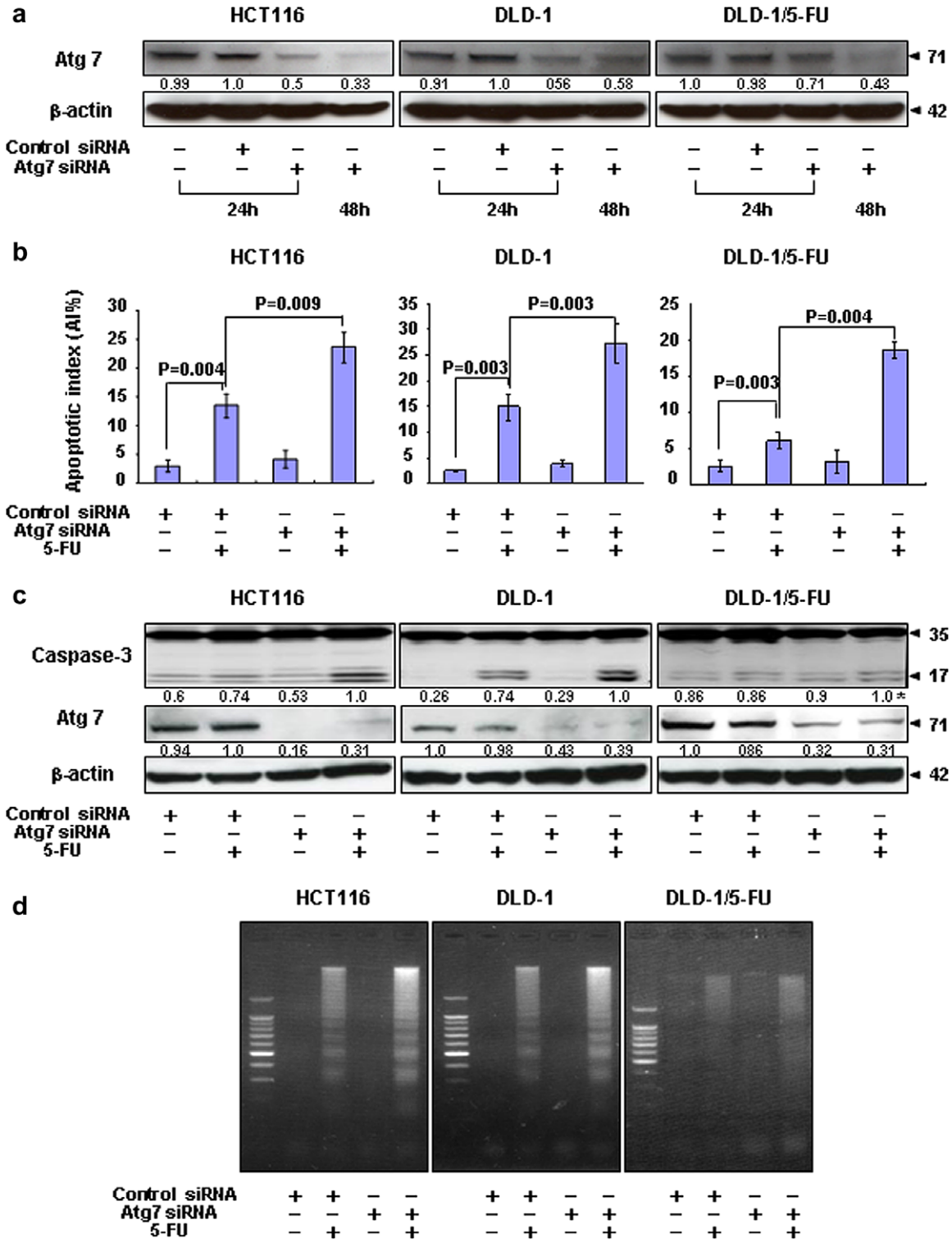

Fig. 3 – Atg7 siRNA increases the 5-FU-induced apoptotic cell death. HCT116, DLD-1, and DLD-1/5-FU cells were transfected with control siRNA or Atg7 siRNA. (a) Immunoblotting was performed to assess the knockdown. (b) Twenty-four hours after transfection, cells were treated with 5-FU (7 μM in HCT116, 50 μM in DLD-1, and DLD-1/5-FU) for 48 h or not. Hochest 33342 assay was performed and the apoptosis index (AI, mean ± SEM) was calculated. (c) Cell lysates were prepared and subjected to immunoblotting of caspase-3, Atg7, and β-actin. The values of the band intensity below the figure represent the densitometric estimation of each band normalised by β-actin. *The active form value. (d) DNA fragmentation assays were performed to observe DNA ladder formation. Data are representative of three independent experiments.

3-MA is a well known autophagy inhibitor, which we confirmed in our experiment (Fig. 2). Immunofluorescence analysis by anti-LC3 antibody showed a diffuse pattern (cytoplasm, LC3-I) in the control and 3-MA group. 5-FU treatment altered LC3 distribution to many coarse dots and punctuate staining (Fig. 2a). This LC3-positive punctuate (LC3-II) represents the autophagosome.[7] Addition of 3-MA decreased and weakened these punctuate signals evoked by 5-FU. The change of LC3 protein was also observed by immunoblotting (Fig. 2b). LC3-II (16 kDa) was induced by 5-FU treatment in all of the cells, but 3-MA reduced them about 26%, 47%, and 40% in HCT116, DLD-1, and DLD-1/5-FU cells, respectively. Correspondingly, the level of p62 protein decreased with 5-FU treatment and increased with 3-MA addition. Overall, 5-FU treatment induced the activation of autophagy in colon cancer cells, but addition of 3-MA inhibited this response and enhanced 5-FU-induced colon cancer cell death.

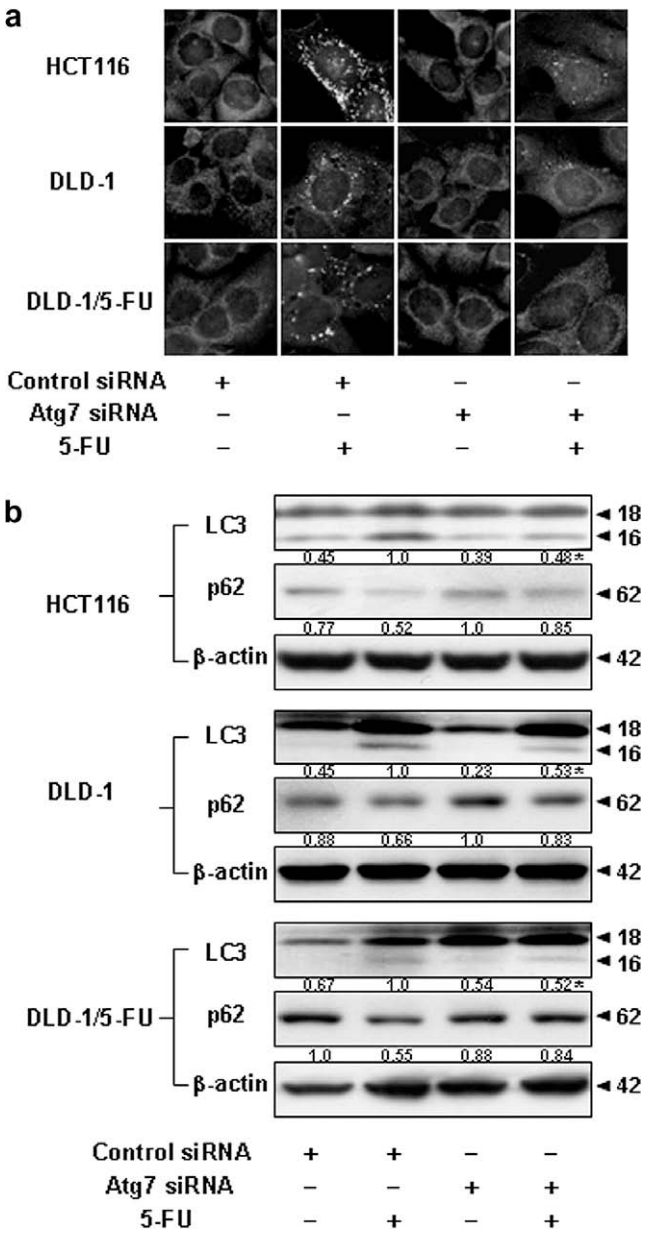

Fig. 4 – Atg7 siRNA decreases 5-FU-induced autophagy. HCT116, DLD-1, and DLD-1/5-FU cells were treated as in Fig. 3. (a) LC3 staining was performed to detect autophagosome formation. (b) Immunoblotting for LC3 and p62 were used to observe autophagy. The values of the band intensity below the figure represent the densitometric estimation of each band normalised by β-actin. *The LC3-II value. Photos (×400) and values are representative of three independent experiments.

3.2. 5-FU-induced apoptosis in colon cancer cell is augmented by Atg7 siRNA

To observe the influence of autophagy more directly, we used Atg7-targeting siRNA to decrease endogenous Atg7 expression. Atg7 is a critical protein during autophagosome formation.[24] The change of 5-FU effect was also examined (Fig. 3). Cell lysates were prepared at 24 h after transfection with Atg7 siRNA or the universal control siRNA. Via immunoblotting, compared with the control, endogenous Atg7 was reduced by 50%, 44%, and 27.6% in HCT116, DLD-1 and DLD-1/5-FU cells, respectively, and it further decreased at 48 h after transfection with Atg7 siRNA (Fig. 3a). 5-FU-induced apoptosis was examined by Hoechst 33342 staining and the apoptotic index (AI) was calculated (Fig. 3b). 5-FU induced nuclear condensation and fragmentation. Of the total cell count, 11.29%, 15.47%, and 6.63% of HCT116, DLD-1, and DLD-1/5-FU cells were apoptotic. With the decrease of Atg7, apoptotic cell death by 5-FU was increased: AI increased to 23.66% in HCT116, 27.14% in DLD-1, and 18.61% in DLD-1/5-FU. Next, apoptosis was also examined by immunoblotting of caspase-3 (Fig. 3c). 5-FU-induced caspase-3 cleavage into its active forms and the decrease of Atg7 increased the active forms in the 3 colon cancer cell types. Furthermore, a DNA fragmentation assay was performed to detect apoptosis (Fig. 3d). In HCT116 and DLD-1 cells, 5-FU caused formation of the DNA ladder and use of the Atg7 siRNA combination increased it. In DLD-1/5-FU cells, the DNA ladder by 5-FU was small but was then enhanced by Atg7 siRNA. Similarly, the change in autophagic activity was examined (Fig. 4). 5-FU-induced punctuate staining of LC3-II was reduced by Atg7 siRNA (Fig. 4a), and 5-FU–induced LC3-II increase was also decreased by Atg7 siRNA about 52%, 47%, and 48% in HCT116, DLD-1, and DLD-1/5-FU cells, respectively (Fig. 4b). The p62 protein level changed correspondingly, decreasing upon 5-FU treatment and increasing upon the addition of Atg7 siRNA. These results suggest that 5-FU-induced colon cancer cell apoptosis was augmented by Atg7 siRNA.

3.3. Anticancer effect of 5-FU and 3-MA combination against DLD-1 xenografts

Next we explored whether the synergistic effect of 3-MA and 5-FU is actually applicable *in vivo*. Nude mice with DLD-1 xenografts were divided into four groups: control, 3-MA, 5-FU, and combination group with intraperitoneal injection of saline, 3-MA (24 mg/kg), 5-FU (30 mg/kg), and 3-MA (24 mg/kg) and 5-FU (30 mg/kg) combination, respectively, at 10, 15, 20, 25, and 30 d. All of the mice were healthy and there were no differences in body weight between groups (Fig. 5a). Compared with the control, 5-FU treatment suppressed the tu-

mour growth and 5-FU combined with 3-MA further suppressed tumour growth (Fig. 5b). Treatment with 3-MA alone had no significant influence on tumour growth. On day 35, mean tumour volume and mean tumour weight of combination group mice were significantly reduced by 66.8% and 49.3% compared with the 5-FU group (Fig. 5b and c). Moreover, the result of immunoblotting of caspase-3, LC3, and p62 from the tumour xenografts extract showed that combination therapy increased activated caspase-3 12.4% compared with treatment of 5-FU alone, and that the LC3-II increase decreased, p62 decrease increased as expected (Fig. 5d). These results demonstrate that use of the 5-FU, 3-MA combination also improves the effect of 5-FU on colon cancer *in vivo*.

3.4. *Bcl-xL, a link between apoptosis and autophagy*

Finally, since it is strongly suggested that there is crosstalk between 5-FU-induced apoptosis and autophagy, we checked expression of the Bcl-2 family which is reportedly implicated in both apoptosis and autophagy.[25] By immunoblot analysis, we found that 5-FU increased the expression of Bcl-xL in both the DLD-1 cell lysate and the DLD-1 tumour xenograft extract. Use of 3-MA combination or Atg7 siRNA combination reduced the increase of Bcl-xL. There had no significant changes in other Bcl-2 family proteins (Fig. 6a). As a result, Bcl-xL appears to be a link between autophagy and the apoptosis pathways in our experiment.

3.5. *P53-AMPK-mTOR may participate in 5-FU-induced autophagy response*

On the other hand, since 5-FU-induced autophagy is important, what is its underlying mechanism? Using immunoblotting, we found that with 5-FU treatment, expression and phosphorylation of tumour suppressor p53 and AMP-activated protein kinase (AMPK) increased significantly and that the mammalian target of rapamycin (mTOR), a gatekeeper of autophagy[18] decreased and the phosphorylation of p70S6K, an indicative of mTOR activity also decreased in the DLD-1 cell lysate and tumour xenograft extract. Use of 3-MA combination or Atg7 siRNA combination reversed these changes as responses to autophagy inhibition (Fig. 6b).

4. Discussion

In colorectal cancer, chemotherapy is currently used to reduce tumour recurrence and prolong patients' lives. However,

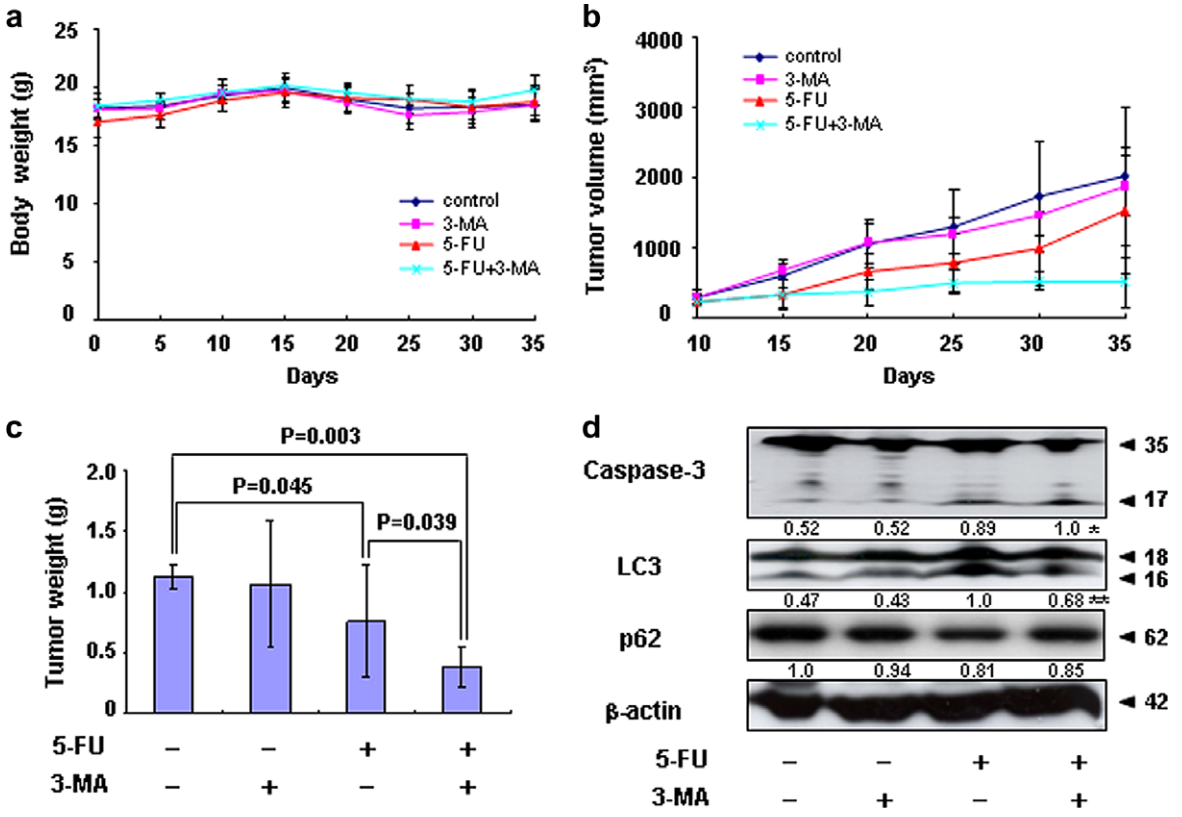

Fig. 5 – Combination therapy of 3-MA and 5-FU mediates tumour suppression. DLD-1 cells were transplanted into nude mice. After 10 d, mice were divided into four groups: control, 3-MA (24 mg/kg), 5-FU (30 mg/kg), and combination (3-MA (24 mg/kg) and 5-FU (30 mg/kg)) and treated on days 10, 15, 20, 25, and 30. (a) Body weights of all mice were measured every 5 d from day 0 to day 35. (b) Tumour progression of all DLD-1 xenografts was evaluated by measurement of tumour volume every 5 d. (c) After 35 d, the tumour xenografts were excised and the tumour weights were measured. (d) Extracts of the tumour xenografts were prepared and subjected to immunoblotting analysis for caspase-3, LC3, p62, and β-actin. The values of the band intensity below the figure represent the densitometric estimation of each band normalised by β-actin. *The active form value. **The LC3-II value. Data represent the means of three independent experiments.

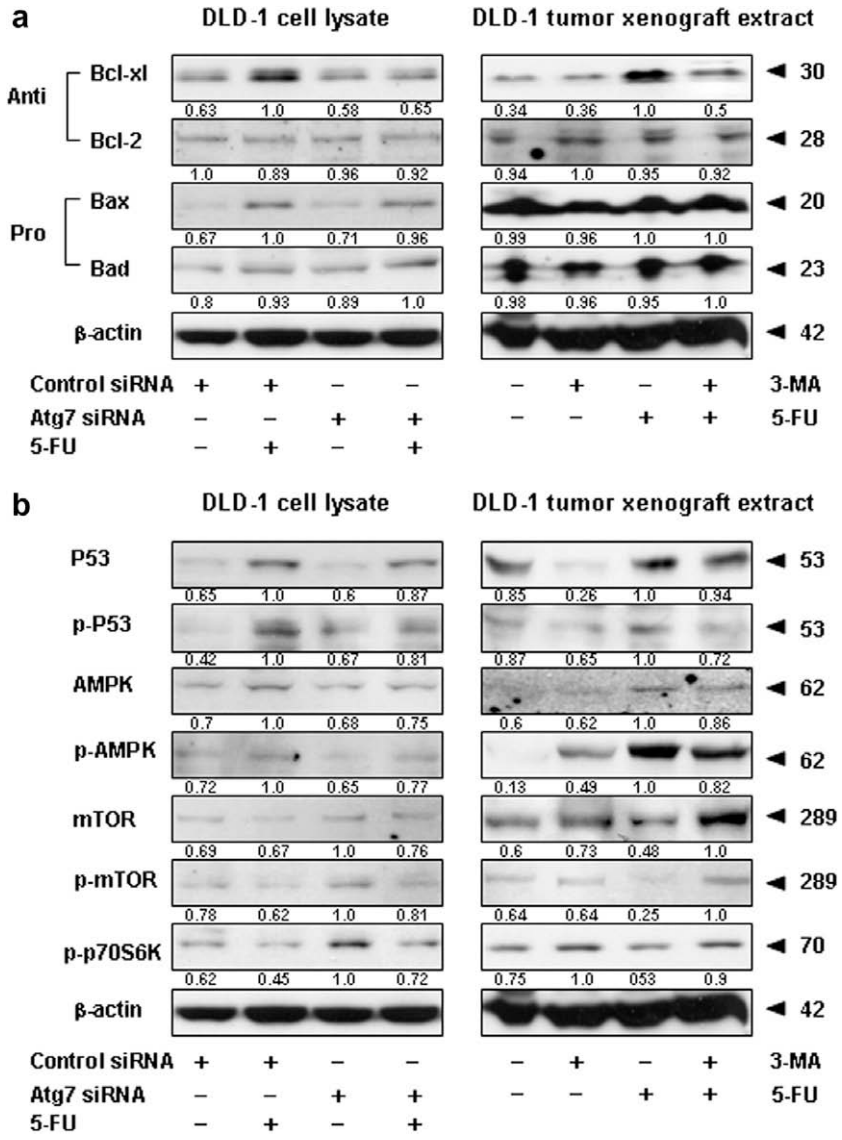

Fig. 6 – Changes in the Bcl-2 family and the p53-AMPK-mTOR proteins. The DLD-1 cell lysate treated as in Fig. 3 and the DLD-1 tumour xenograft treated as in Fig. 5 were prepared and subjected to immunoblotting analysis for Bcl-xl, Bcl-2, Bax, Bad, and β-actin (a). Changes in p53, p-p53, AMPK, p-AMPK, mTOR, p-mTOR, and p-p70S6k were also detected (b). The values of the band intensity below the figure represent the densitometric estimation of each band normalised by β-actin. Data represent the means of three independent experiments.

due to drug resistance, exploration of new chemotherapy strategies is very important. From our previous report,[20] we found that the autophagy inhibitor 3-MA could enhance 5-FU–induced HT29 (a human colon cancer cell line) and colon26 (a mouse colon cancer cell line) cell death (including apoptosis). In this study, we used 2 other human colon cancer-derived cell lines (HCT116 and DLD-1) and 1 5-FU-resistant sub-line (DLD-1/5-FU). As expected, 3-MA inhibited 5-FU–induced autophagy and enhanced 5-FU-induced cell apoptosis in these human primary colon cancer cells, even in 5-FU-resistant DLD-1/5-FU cells. Moreover, this synergistic effect of 3-MA and 5-FU was further demonstrated using the DLD-1 xenograft tumour model *in vivo*. As a result, we propose that 3-MA could be a potential adjuvant agent with 5-FU in the treatment of primary colon cancer.

3-MA is known as an autophagy inhibitor that inhibits the activity of type III PI3K (a kinase that is essential for vesicle nucleation, the first phase of autophagosome formation). Recently, many studies have shown that besides maintaining intracellular homeostasis, autophagy is also implicated in many pathological conditions including cancer.[10] Inducers or inhibitors of autophagy have been reported working well with chemotherapeutic drugs.[23,26–29] However, pharmacological intervention may have functions other than inhibiting autophagy and may regulate other enzymes related to cell viability.[30] We used Atg7 siRNA to genetically inhibit autophagy and then evaluated the role of autophagy more directly. Atg7 is an ubiquitin (E1)-like activating enzyme that is critical to the modification of Atg12-Atg5-Atg16 complex and Atg8-PE (LC3-II in mammals), two important steps during autophago-

some elongation and sequestration.[24] We found that silencing-Atg7 protein reduced 5-FU-induced autophagy and also enhanced 5-FU-induced apoptosis in colon cancer cells. It is noteworthy that the increase of 5-FU-induced caspase-3 activation and apoptosis by Atg7 siRNA seemingly less than the increase by 3-MA, especially in DLD-1/5-FU. This may be due to the fact that 3-MA can also inhibit class I PI3K and thereby inhibit the anti-apoptotic Akt/PKB pathway. In spite of this, our data suggest that 5-FU-induced autophagy plays a protective role from apoptosis in colon cancer and that inhibiting autophagy might be a potential way to improve the effect of 5-FU chemotherapy in colon cancer patients.

The crosstalk between apoptosis and autophagy is quite complex but is a key factor in the outcome of death-related pathologies such as cancer.[31] At the molecular level, the crosstalk between them is manifested by numerous genes including Atg5, the Bcl-2 family, p53, ARF, DAPk, and E2F1.[25,31–33] In our report, Bcl-xL, an anti-apoptosis protein in the Bcl-2 family, was up-regulated by 5-FU and was decreased by both 3-MA and Atg7 siRNA. Bcl-xL seems to act as a crosstalk link between 5-FU-induced apoptosis and autophagy in colon cancer cells. The anti-apoptotic proteins Bcl-2/Bcl-xL also have been reported to be related to autophagy by interaction with Beclin 1 in mice embryonic fibroblasts (MEFs) cells.[25] More detailed investigations are required for understanding the dynamics of the Bcl-2 family relationship with autophagy and apoptosis.

Until now, the known signals that regulate autophagy are almost associated with mTOR, a gatekeeper that is located upstream of the autophagy execution machinery and suppresses autophagy.[19] In cancer cells, mTOR is continually activated by some mutations and autophagy is always suppressed to reduce catabolic activities to favour cancer cell growth and proliferation.[34] The upstream of mTOR contains the AMPK pathway, the marker of cellular energy metabolism, which inhibits mTOR activity upon starvation and calcium signals.[19,35] Recently, p53 tumour suppressor was also reported to positively regulate autophagy via activation of AMPK, subsequent inhibition of mTOR, and required TSC1/2.[36] Ju and colleagues showed that 5-FU chemotherapy caused genotoxic stress in colon cancer cells and then increased p53 expression at the translational level.[37] Consistent with this, our data showed that expression and phosphorylation of p53 was increased by 5-FU treatment, followed by very prominent AMPK increase (both expression and phosphorylation). Correspondingly, mTOR level and activity decreased and autophagy was activated. Inhibition of autophagy by 3-MA or Atg7 siRNA blocked autophagosome formation (autophagy execution system) and led to autophagy inhibition. Then, the upstream mTOR, AMPK, and p53 respond accordingly to retain the autophagy inhibition. These data suggest that p53, AMPK, and mTOR closely associate with the status of autophagy. So, although it is certain that there are other pathways, the p53-AMPK-mTOR pathway might be one of the mechanisms involved in 5-FU–induced autophagy in colon cancer cells.

The combination treatment of 5-FU and 3-MA described in this study was assessed as a useful approach to anti-cancer treatment by both *in vitro* and *in vivo* experiments. In conclusion, our results indicate that in human colon cancer, 5-FU chemotherapy causes autophagy activation to protect cancer cells from apoptosis. Correspondingly, autophagy inhibition could have promising therapeutic potential as 5-FU-based adjuvant chemotherapy against colon cancer.

Conflict of interest statement

None declared.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.02.021.

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