Effects of Ellagic Acid on Chemosensitivity to 5-Fluorouracil in Colorectal Carcinoma Cells

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Abstract. *Ellagic acid has been demonstrated to inhibit the growth of several types of cancer cells. However, whether it sensitizes human colorectal carcinoma cells to 5-fluorouracil, has not yet been investigated. Colorectal carcinoma HT-29, Colo 320DM, SW480 and LoVo cells were treated with ellagic acid (2.5-25 μg/ml) and 5-fluorouracil (5-25 μM) alone and in combination and then the viability was assessed by trypan blue exclusion, apoptosis by annexin-V labeling, mitochondria membrane potential by staining with rhodamine 123, and changes in the levels of proteins involved in apoptosis by immunoblotting. Ellagic acid and 5-fluorouracil synergistically inhibited cell proliferation of HT-29, Colo 320DM and SW480 cells, but cytotoxicity toward LoVo cells seems not to be potentiated by this combination. The combination also elevated apoptotic cell death of HT-29 and Colo 320DM cells. The mitochondria membrane potential was lost in combination-treated HT-29 cells, due to increased B cell lymphoma 2-associated protein X (BAX): B cell lymphoma 2 protein (BCL-2) ratio and caspase-3 activity. Ellagic acid synergistically potentiated chemosensitivity to 5-fluorouracil in at least three colorectal cancer cell lines. The results indicate that ellagic acid has potential as a novel agent sensitizing colorectal cancer cells to 5-fluorouracil.*

In Taiwan, 12,488 patients were newly-diagnosed with colorectal carcinoma (CRC) in 2009, making it the most common type of cancer mationwide (1). Recent advanced surgical and chemotherapeutic technologies have reduced the lethality of CRC. 5-Fluorouracil (5-FU) is the most widely used chemotherapeutic agent for the treatment of CRC. The greatest challenge in 5-FU treatment of CRC is chemoresistance of CRC cells and novel strategies to overcome this are the primary aim of investigation (2). Polyphenols may be potential candidates which increase sensitivity of CRC to 5-FU (3, 4). Ellagic acid (EA) is a polyphenol widely found in berries, walnuts, pecans, pomegranate, cranberries and longan. EA is well-known to have free radical-scavenging activity and has been approved in Japan as an existing food additive for antioxidative purposes (5). A recent study demonstrated that EA exerted no remarkable chronic toxic effect on F344 rats (6). Furthermore, EA exhibits potent anticancer and anticarcinogenic activity in colorectal, prostate, esophagial and breast cancer (7). However, there is no evidence for the potential effect of EA in the sensitization of CRC cells to 5-FU.

Materials and Methods

Materials. Roswell Park Memorial Institute (RPMI) media 1640, minimum essential medium (MEM), fetal bovine serum (FBS), L-glutamine, trypsin and antibiotics were purchased from Gibco Ltd. (Paisley, UK). EA, proteinase inhibitor cocktail, sodium orthovanadate, sodium fluoride, sodium pyrophosphate, Triton X-100, ammonia persulfate, rhodamine 123, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and Tween 20 were from Sigma (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL, USA). Acrylamide was from Bio-Rad (Hercules, CA, USA). Polyvinylidene difluoride (PVDF) membrane (Immobilon-P) was from Millipore (Bedford, MA, USA). Mouse monoclonal antibodies against caspase-3 and B-cell lymphoma 2 (BCL-2) were from Zymed (San Francisco, CA, USA). Goat polyclonal antibodies against poly (ADP-ribose) polymerase (PARP) and Bcl-2-associated protein X (Bax) and goat anti-rabbit, anti-mouse and rabbit anti-goat secondary antibodies conjugated with horseradish peroxidase (HRP) were from R&D

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Systems (Minneapolis, MN, USA). X-ray films were from Fuji (Tokyo, Japan). Annexin V conjugated with fluorescein isothiocyanate (FITC) was from Becton Dickinson (Franklin Lakes, NJ, USA).

Cell lines. Human CRC cell lines SW480, Colo 320DM, LoVo and HT-29 were obtained from the Bioresource Collection and Research Center, Taiwan. SW480 cells were established from a primary adenocarcinoma of a patient with Duke's stage B colon cancer. Colo 320DM cells were derived from a moderately-differentiated colonic adenocarcinoma of a patient. LoVo cells were established from a fragment of a metastatic tumor nodule in the left supraclavicular region of a patient with colon cancer. HT-29 cells were derived from the tumor of a patient with grade II colonic adenocarcinoma. All cells used here were incubated at 37˚C with the media, in a water-saturated atmosphere as described by our previous report (8). All experiments were carried out on cell lines passaged 5-20 times.

Cell proliferation assay. CRC cell lines were plated at 100,000 cells in six-well tissue culture dishes. After 18 h of culture, cells were treated with different concentrations of EA dissolved in dimethyl sulfoxide (0, 2.5, 10, or 25 μg/ml), concentrations selected according to a previous report (9), or 5-FU (0, 5, 10, or 25 μM) or a combination of both drugs. At different time points, cells were collected by trypsinization, stained with trypan blue, and the cell number in suspension was counted in duplicate using a hemocytometer. The combination effect was calculated according to the equation as described by Kern *et al*. (10). An expected value of the cell survival, S-exp, was defined as the product of the survival observed for EA-alone and the survival observed for 5-FU-alone: S-exp=(S-EA)×(S-5-FU)

The actual survival observed for the combination of EA and 5-FU was defined as S-obs. A synergistic ratio, R, was calculated as: R=(S-exp)/(S-obs)

Synergy was defined as any value of R greater than unity. Values of R of 1.0 indicated an additive effect. An R value less than 1.0 revealed an absence of synergy.

Apoptosis analysis. Apoptosis measurement of treated and untreated cells was carried out using annexin V to label cell surface phosphatidylserine of apoptotic cells, as described by Hsu *et al*. (11). Briefly, treated cells were trypsinized and washed twice with phosphate-buffered saline, then suspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Cells were stained with a final concentration of 2 μg/ml of annexin V conjugated with FITC at room temperature in the dark for 30 min. Flow cytometry (Becton Dickinson, CA, USA) measured fluorescence intensity with the FL-1H channel detecting FITC. Untreated cells served as the negative control.

Mitochondrial membrane potential ($\Delta\psi_m$). $\Delta\psi_m$ measurement was carried out essentially as described by Hsu *et al*. (11). Briefly, treated and untreated cells were harvested and suspended at a density of 1×10^6 cells/ml in fresh medium. The cells were then stained with rhodamine 123 at a final concentration of 10 μg/ml for 30 min at 37˚C. Cells were then washed twice with fresh medium and the fluorescence intensity of cells was immediately examined by flow cytometry. Ten thousand cells without cell debris were analyzed and the rhodamine 123-negative cells were defined as those with a lower fluorescence intensity than untreated cells.

Table I. *The combination index (R value*) of 5-flueouracil (5-FU) and ellagic acid (EA) for colorectal cancer cell lines*.

		5-FU (μM)		
Cell line	Ellagic acid (μg/ml)	5	10	25
HT-29	2.5	1.09	1.00	1.03
	10	1.13	1.14	1.23
	25	1.15	1.02	1.19
Colo 320DM	2.5	1.03	1.15	1.08
	10	1.03	1.21	1.19
	25	1.03	1.05	1.04
SW480	2.5	1.01	0.84	0.90
	10	1.20	1.07	1.13
	25	1.17	1.21	0.99
LoVo	2.5	0.91	0.68	0.79
	10	0.72	0.60	0.69
	25	0.86	0.67	0.79

*The combination effect was calculated according to the equation as described by Kern *et al*. (10). An expected value of the cell survival, S-exp, was defined as the product of the survival observed for EA-alone and the survival observed for 5-FU-alone: S-exp=(S-EA)×(S-5-FU). The actual survival observed for the combination of EA and 5-FU was defined as S-obs. A synergistic ratio, R, was calculated as: R=(S-exp)/(S-obs). Synergy was defined as any value of R greater than unity. Values of R of 1.0 or less indicated an absence of synergy.

Immunoblotting. Treated cells were washed with ice-cold phosphate-buffered saline and lysed in homogenization buffer (10 mM Tris-HCl at pH 7.4, 2 mM EDTA, 1 mM EGTA, 50 mM NaCl, 1% Triton X-100, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1:100 proteinase inhibitor cocktail) on ice for 30 min. After centrifugation for 30 min at 100,000 ×*g* at 4˚C to remove insoluble materials, the protein concentration of the lysate was determined by the BCA protein assay kit and proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved bands were electrotransferred to PVDF membranes using a semi-dry blot apparatus (Bio-Rad). Immunoblotting was performed by incubating PVDF membranes with 5% non-fat milk in Tris-buffered saline supplemented with Tween 20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween 20) for 1 h at room temperature for blocking the residue-free protein binding sites on PVDF. The membrane was incubated with different primary antibodies in 3% non-fat milk in TBST at 4˚C for 18 h. After repeated washing with TBST, the membrane was incubated with secondary antibodies conjugated with HRP. Immunoblots were developed using enhanced chemiluminescence and the luminescence was visualized on X-ray film or a chemoluminescence detection system (Bio-Rad).

Statistical analysis. All data are the average from three independent experiments and expressed as means±standard deviation (SD) unless stated otherwise. Differences between groups were calculated using the Student's unpaired *t*-test. $p<0.05$ was considered statistically significant. All statistical analysis were performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA).

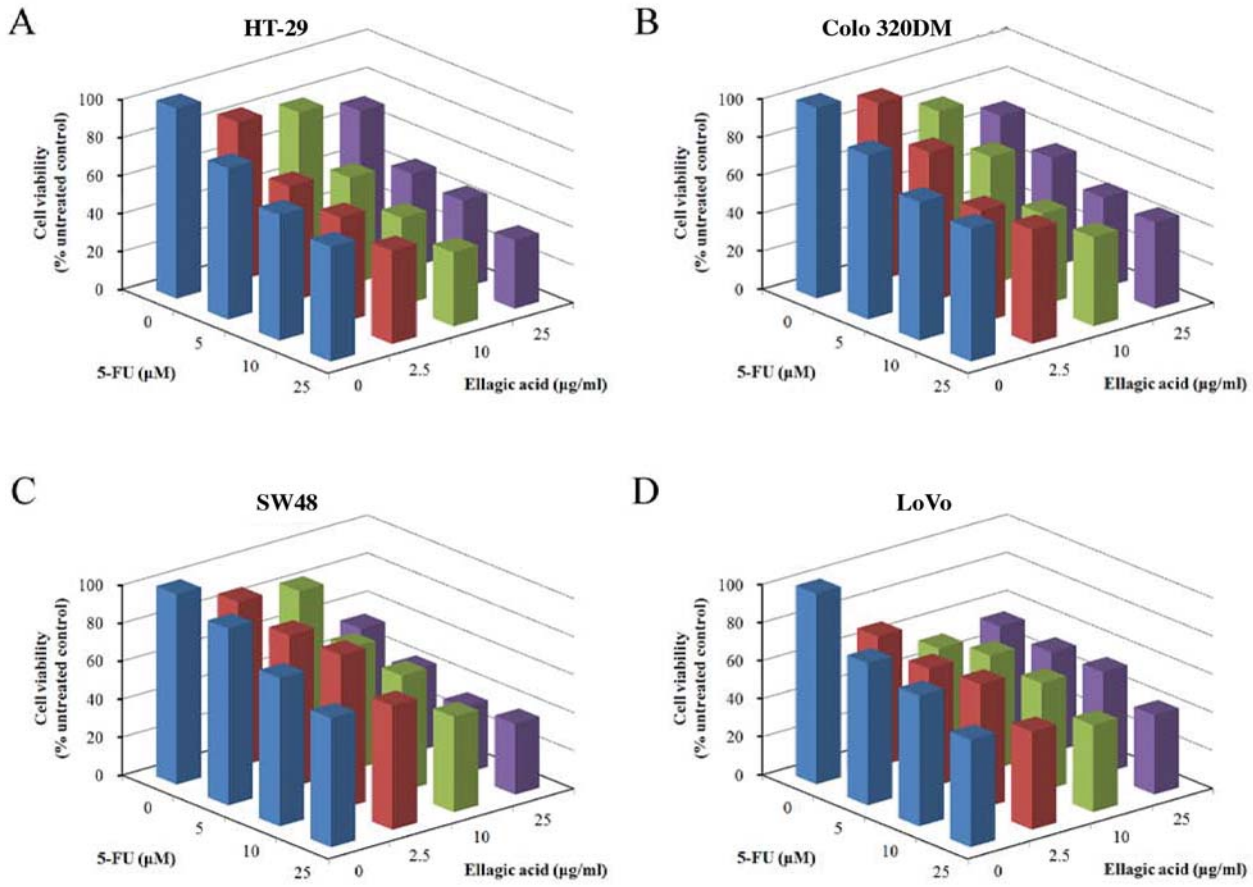

Figure 1. *The combination effect of ellagic acid (EA) and 5-fluorouracil (5-FU) on colorectal cancer cells. A total of 100,000 HT-29 (A), Colo 320DM (B), SW480 (C) and LoVo cells (D) in 6-well plates were treated with increasing concentrations of EA, 5-FU, or their combination as indicated at 37˚C for 24 h. Viable cells were trypsinized, stained with trypan blue and counted under a microscope. Data are the average of three independent experiments and expressed as means±SD.*

Results

Synergistic cell proliferation inhibition by combination of EA and 5-FU. We combined each concentration of 5-FU (5, 10, 25 μM) with EA (2.5, 10, 25 μg/ml) to treat four CRC cell lines. Inhibition of proliferation by combination treatment of HT-29 cells was from 36.1% to 60.9%, whereas that for EA and 5-FU treatments alone was 71.0% to 83.6% and 60.4% to 79.6%, respectively (Figure 1A), synergistic effects were apparent as all of the R values were greater than unity (Table I). Proliferation inhibition of Colo 320DM cells by the combination treatments, compared with untreated cells, was from 45.4% to 78.4%, whereas that for EA and 5-FU treatments alone was 68.2% to 93.3% and 69.6% to 86.2%, respectively (Figure 1B). Calculation of the combination index R showed that the values for all combination groups were greater than unity, demonstrating the synergistic effect of EA and 5-FU in Colo 320DM cells (Table I). The inhibition by combination treatment on SW480 cells was from 37.3% to 80.2%, whereas that for EA and 5-FU treatments alone was 54.2% to 86.7% and 68.0% to 93.1%, respectively (Figure 1C). Some of the combinations had synergistic effects but antagonistic effects were also observed (Table I). Combination treatment of LoVo cells inhibited cell proliferation by 41.6% to 61.4%, whereas that for EA and 5-FU treatments alone was 55.1% to 68.3% and 56.4% to 74.5%, respectively (Figure 1D), demonstrating antagonistic effects as all of the R values were less than unity (Table I).

The potentiation of apoptosis of CRC cells by the combination of 5-FU and EA. Phosphatidylserine translocation was assessed to determine apoptosis of combination-treated CRC cells by staining with FITC-conjugated annexin V. The proportion of Annexin V-positive HT-29 cells increased at 25 μg/ml EA, or

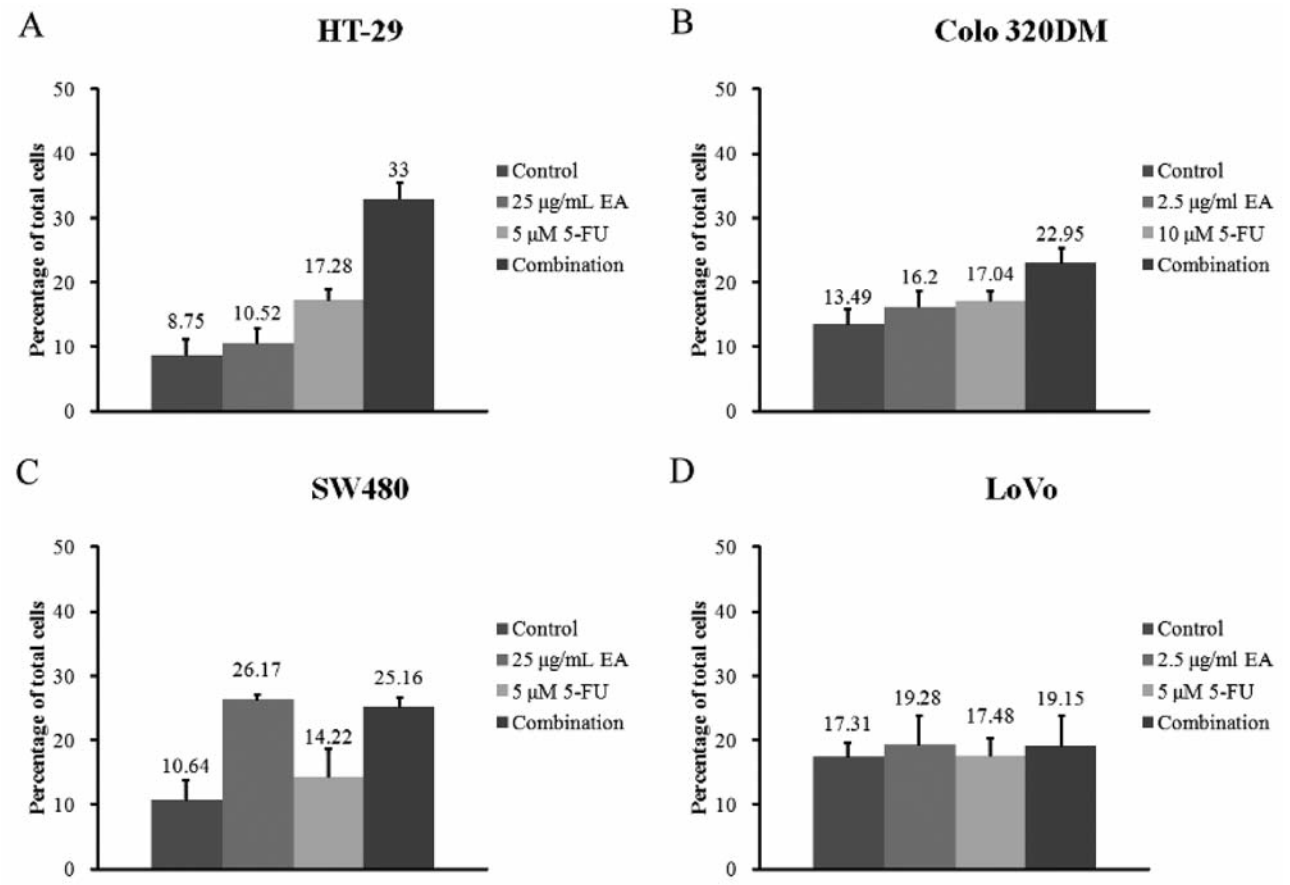

Figure 2. *Comparing apoptosis induced by ellagic acid (EA)- and 5-fluorouracil (5-FU)-alone, and by their combination in colorectal cancer cells. HT-29 (A), Colo 320DM (B), SW480 (C) and LoVo cells (D) were treated with EA or 5-FU, or their combination at 37˚C for 24 h. The treated cells were then suspended and stained with annexin V conjugated with fluorescein isothiocyanate and analyzed by flow cytometry. The reported data are the averages of three independent experiments and are expressed as means±SD.*

5 μM 5-FU with 10.52% and 17.38% of total cells. The proportion of apoptotic cells dramatically increased to 33% of total cells with the combination of both drugs (Figure 2A; $p<0.05$). In Colo 320DM cells, the combination treatment also produced more apoptotic cells than either drug alone (Figure 2B). Similar apoptosis-inducing effects were found with EA alone and its combination with 5-FU in SW480 cells (Figure 2C). There was no remarkable increase in apoptotic LoVo cells by EA, 5-FU or their combination (Figure 2D).

Loss of $\Delta\psi_m$ in EA plus 5-FU-treated HT-29 cells. To investigate whether the apoptosis induction by combination of EA with 5-FU in HT-29 cells involved the loss of mitochondrial integrity, the $\Delta\psi_m$ of treated cells was analyzed. The percentage of rhodamine 123-negative cells in the control group was 7.4% of total cells. After treatment with EA or 5-FU alone, this percentage was 12.99% in 25 μg/ml EA-treated cells or 8.14% in 5 μM 5-FU-treated cells. When EA was combined with 5-FU to treat HT-29 cells, the percentage of rhodamine 123-negative cells increased to 39.62% of total cells.

Elevated BAX:BCL-2 ratio in combination-treated HT-29 cells. The expression of the inactive form of caspase-3 was slightly reduced by EA, 5-FU and their combination. The expression of the active form of caspase-3 (cleaved caspase-3) in HT-29 cells was slightly elevated by EA, and by 5-FU treatment, but was dramatically increased by their combination (Figure 3A). PARP, which is an intracellular substrate of the active form of caspase-3, was remarkably cleaved in combination-treated cells. The level of anti-apoptosis oncoprotein BCL-2 was suppressed by EA, 5-FU, and the combination treatment to HT-29 cells, whereas that of the apoptosis-promoting protein BAX was elevated (Figure 3B). The BAX:BCL-2 ratio was highest for the combination-treated HT-29 cells than for EA or 5-FU alone (Figure 3C). These results indicate that activation of apoptosis in HT-29 cells by the combination of EA and 5-FU was mainly due to increase in the BAX:BCL-2 ratio.

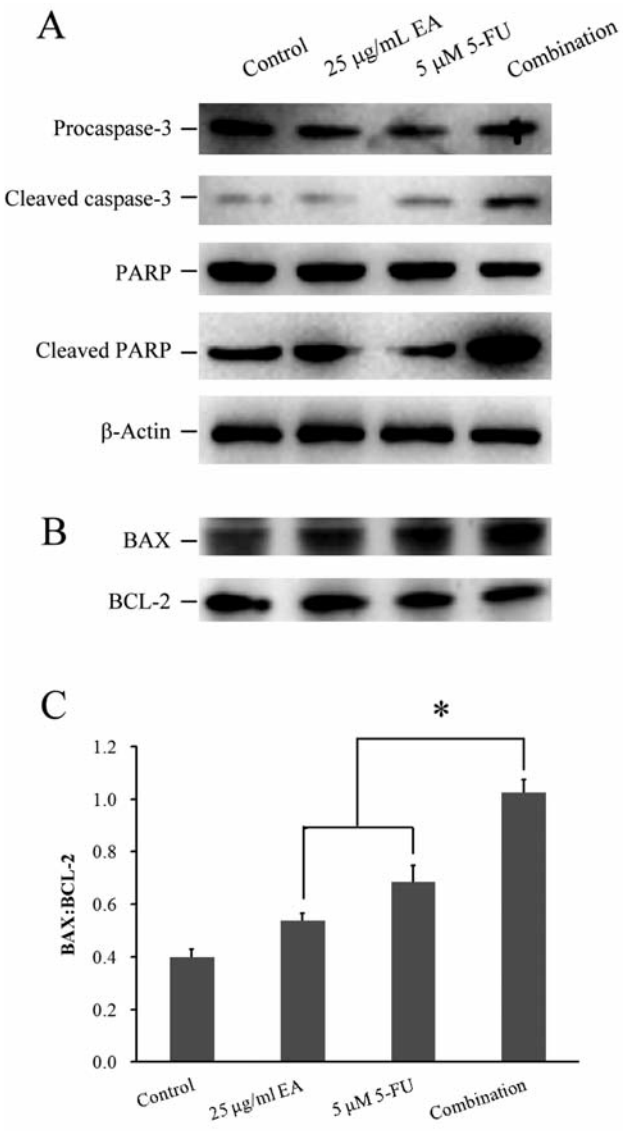

Figure 3. *The levels of apoptosis-associated proteins in treated HT-29 cells. Cell protein lysates from treated HT-29 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and immunoblotted for pro-caspase-3, cleaved caspase-3, poly (ADP-ribose) polymerase (PARP), and cleaved PARP (A), and B-cell lymphoma protein-2 (BCL-2) and BCL-2 associated protein X (BAX) (B). Protein levels were quantified and normalized using the density of untreated control and the BAX:BCL-2 ratio was calculated (C). *p<0.05.*

Discussion

In the current study we investigated the effects of EA on the chemosensitization of CRC cell lines to 5-FU and found a synergistic effect on at least three cell lines, HT-29, Colo 320DM and SW480. The main reason for this effect may be the enhanced apoptosis induction by the combination of EA and 5-FU, at least in HT-29 cells. The apoptosis induced by combination of EA and 5-FU may occur through the mitochondria pathway in HT-29 cells, in which the mitochondria membrane potential was lost and the BAX:BCL-2 ratio was elevated. To the best of our best knowledge, this is the first report that has revealed the 5-FU-chemosensitizing effect of EA on CRC cells.

The combination effect of EA and 5-FU seems to be associated with the sensitivity to EA in CRC cells. Colo 320DM and HT-29 cells were less sensitive to EA whereas SW480 and LoVo cells seemed to be more sensitive to EA. The R value in LoVo cells, for example, was lower than unity, which demonstrates no synergistic effect, even at the combination with the highest concentrations of EA and 5-FU. In contrast, the R value in HT-29 cells was higher than unity and the survival rate of combination-treated cells was lower than 40%. The main reason for this may be the apoptosis induction. The combination-treated HT-29 and Colo 320DM cells with 5-FU and EA showed more apoptotic cells than drug-alone treated cells, while the apoptotic cells in LoVo cultures were unremarkably changed between drug-alone and combination-treated cells. The results indicate that the apoptotic induction may sensitize colorectal cancer cells to chemotherapy.

HT-29 cells were more sensitive to the 5-FU and EA combination, partly due to apoptosis enhancement. One of the apoptosis pathways is controlled by mitochondria permeability. Previous studies reported that the mechanism of naturally-occurring phytochemical-induced apoptosis in CRC cells involves mitochondria (8, 11, 12). In the present study, we evaluated the combination-treated HT-29 cells for staining by rhodamine 123, which is sequestered by living cells with normal $\Delta\Psi_m$. The untreated control groups of HT-29 cells showed strong fluorescence on flow cytometry, whereas treatment with EA or 5-FU alone only slightly influenced cell $\Delta\Psi_m$. By contrast, the $\Delta\Psi_m$ was lost after combination treatment, indicating that the combination treatment-induced apoptosis in HT-29 cells was mediated by a mitochondrial pathway. The BCL-2 family of proteins is important in mediating apoptosis in CRC cells (11, 13). Some family members promote apoptosis [*e.g.* BAX and Bcl-2-associated death promoter (BAD)] while others inhibit it (*e.g.* BCL-2 and BCL-X). These proteins form multimers which act as pores in cell membranes, controlling the flux of molecules between cellular compartments. BCL-2 inhibits apoptosis by inhibiting the release of cytochrome-c [apoptotic protease activating factor-2 (APAF-2)] and apoptosis-inducing factor (AIF) from the mitochondria to the cytoplasm and by limiting the activation of caspase-3 through inhibition of its activator protein, APAF-1 (14, 15). In our recent report, the ratio of BAX:BCL-2 protein was found to be the determining factor in transmitting the apoptosis signal, which finally triggers caspase-3 activation

(8). In the present study, the decrease in BCL-2 levels and the elevation of BAX levels in combination-treated HT-29 cells was correlated with apoptosis, indicating that the mechanism of apoptosis induction by the combination of EA and 5-FU in this cell line is mainly due to an increase the BAX:BCL-2 ratio. Our results also showed that in HT-29 cells, the combination of EA and 5-FU concomitantly led to self-cleavage of pro-caspase-3, which was theoretically activated, leading to cleavage of one of its substrate, PARP. This may explain the synergistic combinatorial effect of EA and 5-FU on HT-29 cells.

In conclusion, we here reveal that EA potentiates chemosensitivity of HT-29, Colo 320DM and SW480 CRC cells to 5-FU. One of the possible mechanisms for this is an increase in mitochondria-mediated apoptosis, at least in HT-29 cells. The EA-mediated chemosensitization effect seems to be limited in less sensitive cell lines. EA may thus play a regulatory role in reducing chemoresistance of CRC cells. However, apoptosis may not be the only mechanism for EA-mediated chemosensitization of CRC cells. Other mechanisms rather than apoptosis may be involved and require further investigation.

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References

1 Taiwan Department of Health, Executive Yuan. Health and Vital Statistics. (2009). Available at: http://www.bhp.doh.gov.tw/bhpnet/portal/StatisticsShow.aspx? No=200911300001. Assessed on 5 August, 2012.

2 Réti A, Pap E, Adleff V, Jeney A, Kralovánszky J and Budai B: Enhanced 5-fluorouracil cytotoxicity in high cyclooxygenase-2-expressing colorectal cancer cells and xenografts induced by non-steroidal anti-inflammatory drugs via down-regulation of dihydropyrimidine dehydrogenase. Cancer Chemother Pharmacol *66*: 219-227, 2010.

3 Colin D, Gimazane A, Lizard G, Izard JC, Solary E, Latruffe N and Delmas D: Effects of resveratrol analogs on cell cycle progression, cell cycle associated proteins and 5-fluorouracil sensitivity in human derived colon cancer cells. Int J Cancer *124*: 2780-2788, 2009.

4 Gupta SC, Kannappan R, Reuter S, Kim JH and Aggarwal BB: Chemosensitization of tumors by resveratrol. Ann NY Acad Sci *1215*: 150-160, 2011.

5 HHLW (Ministry of Health, Labor and Welfare of Japan), List of Existing Food Additives, Notification No. 120 of the Ministry of Health and Welfare. Japan, 1996.

6 Tasaki M, Umemura T, Maeda M, Ishii Y, Okamura T, Inoue T, Kuroiwa Y, Hirose M and Nishikawa A: Safety assessment of ellagic acid, a food additive, in a subchronic toxicity study using F344 rats. Food Chem Toxicol *46*: 1119-1124.

7 Jurenka JS: Therapeutic applications of pomegranate (*Punica granatum* L.): A review. Altern Med Rev *13*: 128-144, 2008.

8 Chung YC, Lin CC, Chou CC and Hsu CP: The effect of longan seed polyphenols on colorectal carcinoma cells. Eur J Clin Invest *40*: 713-721, 2010.

9 Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG and Heber D: *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem *16*: 360-367, 2005.

10 Kern DH, Morgan CR and Hildebrand-Zanki SU: *In vitro* pharmacodynamics of 1-beta-D-arabinofuranosylcytosine: Synergy of antitumor activity with cis-diamminedichloroplatinum(II). Cancer Res *48*: 117-121, 1988.

11 Hsu CP, Lin YH, Zhou SP, Chung YC, Lin CC and Wang SC: Longan flower extract inhibits the growth of colorectal carcinoma. Nutr Cancer *62*: 229-236, 2010.

12 Chung YC, Huang CC, Chen CH, Chiang HC, Chen KB, Chen YJ, Liu CL, Chuang LT, Liu M and Hsu CP: Grape seed procyanidins inhibit the *in vitro* growth and invasion of pancreatic carcinoma cells. Pancreas *41*: 447-454, 2012.

13 Rossé T, Olivier R, Monney L, Rager M, Conus S, Fellay I, Jansen B and Borner C: BCL-2 prolongs cell survival after BAX-induced release of cytochrome c. Nature *391*: 496-499, 1998.

14 Umesalma S and Sudhandiran G: Ellagic acid prevents rat colon carcinogenesis induced by 1,2 dimethyl hydrazine through inhibition of AKT-phosphoinositide-3 kinase pathway. Eur J Pharmacol *660*: 249-258, 2011.

15 Reed JC: Balancing cell life and death: BAX, apoptosis, and breast cancer. J Clin Invest *97*: 2403-2404, 1996.

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