MOLECULAR MEDICINE REPORTS 18: 2880-2888, 2018

Corosolic acid reduces 5-FU chemoresistance in human gastric cancer cells by activating AMPK

JUN BEOM PARK[1*], JIN SUN LEE[1,2*], MYUNG SUN LEE[3],
EUN YOUNG CHA[3], SOYEON KIM[3] and JI YOUNG SUL[1,2]

[1]Department of Surgery, Chungnam National University Hospital; [2]Department of Surgery and
Research Institute for Medicinal Sciences, Chungnam National University College of Medicine;
[3]Biomedical Research Institute, Chungnam National University Hospital, Daejeon 35015, Republic of Korea

Received January 31, 2018; Accepted June 27, 2018

DOI: 10.3892/mmr.2018.9244

Abstract. 5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents for gastric cancer. Resistance to 5-FU-based chemotherapy remains the major obstacle in the treatment of gastric cancer. A growing body of evidence has suggested that adenosine monophosphate-activated protein kinase (AMPK) is pivotal for chemoresistance. However, the mechanism by which AMPK regulates the chemosensitivity of gastric cancer remains unclear. In the present study, how corosolic acid enhanced the chemosensitivity of gastric cancer cells to 5-FU via AMPK activation was investigated. A 5-FU-resistant gastric cancer cell line (SNU-620/5-FU^R) was established, which had a marked increase in thymidine synthase (TS) expression but reduced AMPK phosphorylation when compared with the parental cell line, SNU-620. AMPK regulation by 5-aminoimidazole-4-carboxamide ribonucleotide or compound c was revealed to be markedly associated with TS expression and 5-FU-resistant cell viability. In addition, corosolic acid activated AMPK, and decreased TS expression and the phosphorylation of mammalian target of rapamycin/4E-binding protein 1 in a dose-dependent manner. Corosolic acid treatment significantly reduced cell viability while compound c reversed corosolic acid-induced cell growth inhibition. The 5-FU-resistance sensitization effect of corosolic acid was determined by the synergistic reduction of TS expression and inhibition of cell viability in the presence of 5-FU. The corosolic acid-induced AMPK activation was markedly increased by additional 5-FU treatment, while compound c reversed AMPK phosphorylation. In addition, compound c treatment reversed corosolic acid-induced apoptotic markers such as capase-3 and PARP cleavage, and cytochrome c translocation to cytosol, in the presence of 5-FU. Corosolic acid treatment in the presence of 5-FU induced an increase in the apoptotic cell population based on flow cytometry analysis. This increase was abolished by compound c. In conclusion, these results implied that corosolic acid may have therapeutic potential to sensitize the resistance of gastric cancer to 5-FU by activating AMPK.

Introduction

Gastric cancer is one of the most common malignant diseases and is the second leading cause of cancer-related death worldwide. The prognosis for advanced gastric cancer remains poor (1). Early stage gastric cancer patients are asymptomatic or experience minor symptoms (2). Most patients with symptoms that can be noticed clinically have reached an advanced stage (2). Surgery is the only potentially curative treatment for gastric cancer. To improve upon low survival outcomes one of major obstacles in cancer therapy, the development of tumor chemoresistance, needs to be overcome. Therefore, it is necessary to investigate the mechanisms involved in chemoresistance, to develop strategies that sensitize cancer cells to chemotherapeutic agents.

5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents for gastric cancer. Despite its many advantages, clinical applications of 5-FU have been limited by drug resistance that arises due to several factors, including altered drug influx and efflux, enhancement of drug inactivation, and mutations to the drug target (3). Several intracellular enzymes, thymidine synthase (TS), dihydropyrimidine dehydrogenase (DPD), methylenetetrahydrofolate reductase (MTHFR) and thymidine phosphorylase (TP), are considered important predictors for 5-FU sensitivity or resistance (4). However, mechanisms underlying 5-FU anti-tumor activity and drug resistance have not been fully revealed yet.

TS expression and activity are increased in several tumor tissues, including lung, cervical, breast, and gastric cancers. They are considered to be indicators of cell proliferation and are associated with poor prognosis. TS has been used as an

---

Correspondence to: Professor Ji Young Sul, Department of Surgery and Research Institute for Medicinal Sciences, Chungnam National University College of Medicine, 33 Munhwa-ro, Jung-gu, Daejeon 35015, Republic of Korea
E-mail: jysul@cnu.ac.kr

[*] Contributed equally

Key words: gastric cancer, corosolic acid, 5-fluorouracil resistance, adenosine monophosphate-activated protein kinase

important target in chemotherapy (5,6). Several studies have shown that TS expression is a key regulator of 5-FU resistance/sensitivity (7,8).

Adenosine monophosphate-activated protein kinase (AMPK) is a heterodimeric enzyme consisting of a catalytic α-subunit and two non-catalytic β and γ subunits. AMPK functions in the cellular metabolism of glucose, lipid, and protein (9). The role of metformin, an AMPK activator, as a chemosensitizer has been investigated in Bel-7402/5-FU cells (hepatocellular carcinoma) and MCF7/5-FU cells (breast cancer) (10,11). It has been reported that AICAR, another AMPK activator, enhanced the pro-apoptotic effect of 5-FU in 5-FU-resistant SGC-7901 cells (gastric cancer) (12). Additionally, it has been reported that phosphorylated AMPK level is reduced in 5-FU-resistant gastric cancer cells while glucose metabolism is increased in 5-FU-resistant HepG2 cells (13). However, it remains unknown as to whether AMPK can increase chemosensitization in gastric cancer cells.

Corosolic acid (2a-hydroxyursolic acid), one of the main triterpenoids, has been discovered in many medicinal plants such as *Lagerstroemia speciosa* (banaba) and *Weigela subsessilis* (14,15). Corosolic acid not only displays remarkable hypoglycemic effects in some animal experiments and clinical trials (16,17), but has also been shown to possess antitumor effects against several cancers, including liver, colon, lung, and gastric cancer (18-21). Previous studies have reported that corosolic acid can enhance the anticancer effect of 5-FU in SNU-620 and NCI-N87 gastric cancer cells, suggesting that it might act as an AMPK activator (21-25). Among natural chemicals, curcumin, epigallocatechin gallate (EGCG), and sinomenine have been found to be able to sensitize 5-FU resistance in gastric cancers (26-28). However, whether corosolic acid can do the same for 5-FU resistance in cancers remains unclear.

Therefore, the objective of this study was to determine the effect of corosolic acid on the response of gastric cancer to 5-FU. We used 5-FU resistant human gastric cancer cells (SNU-620/5-FU^R) and treated them with corosolic acid in the presence or absence of 5-FU to investigate the effect of corosolic acid on 5-FU resensitization, and determine the mechanism of action.

Materials and methods

Materials. RPMI-1640, fetal bovine serum (FBS) and penicillin/streptomycin were obtained from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Trypsin/EDTA was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The following primary antibodies were used: Rabbit polyclonal anti-human thymidylate synthase (1:1,000; no. 3766), rabbit polyclonal anti-human caspase-3 (1:1,000; no. 9662), rabbit polyclonal anti-human poly-(ADP-ribose) polymerase (PARP) (1:1,000; no. 9542), rabbit polyclonal anti-human AMPK (1:1,000; no. 2532), rabbit monoclonal anti-human phospho-AMPK (Thr172) (1:1,000; no. 2535), rabbit polyclonal anti-human mTOR (1:1,000; no. 2972), rabbit polyclonal anti-human phospho-mTOR (Ser2448) (1:1,000; no. 2971), rabbit polyclonal anti-human 4E-binding protein 1 (4EBP1) (1:1,000; no. 9452) and rabbit polyclonal anti-human phospho-4EBP1 (Thr70) (1:1,000; no. 9455) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), and rabbit polyclonal anti-human GAPDH (1:1,000; sc-25778) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Transduction Lab (Lexington, KY, USA). SuperSignal® West Pico Chemiluminescent Substrate was purchased from Pierce (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 5-FU was provided by Choongwae Pharmaceutical Co., Ltd. (Seoul, Korea). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan) and the EzWay Annexin-V-FITC Apoptosis Detection kit was purchased from KomaBiotech, Inc. (Seoul, Korea). A Mitochondrial Apoptosis Staining kit was purchased from PromoKine® (PromoCell GmbH, Heidelberg, Germany). Corosolic acid, compound c, AICAR and all other reagents were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture. Human gastric carcinoma SNU-620 cells were purchased from Korean Cell Line Bank (Seoul, Korea). Cells were grown in RPMI-1640 media supplemented with 10% (v/v) FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml) at 37˚C in a humidified CO_2 (5%)-controlled incubator. 5-FU-resistant SNU-620/5-FU^R cells were established by repeated cultures of SNU-620 with constant treatment with 7.5 μM 5-FU.

Cell growth inhibition assay. Cells were seeded at 5x10^3 cells/ml in 96-well microplates and allowed to attach for 24 h. 5-FU (~750 μM) or corosolic acid (~25 μM) were added to the medium at various concentrations. Following treatment, the cell cytotoxicity and/or proliferation was assessed using the CCK-8 assay. Briefly, highly water-soluble tetrazolium salt [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], produced an orange-colored water-soluble product, formazan. The quantity of formazan dye generated by dehydrogenases in the cells was directly proportional to the number of living cells. CCK-8 (10 μl) was added to each well and incubated for 3 h at 37˚C; cell proliferation and cytotoxicity were assessed by measuring the absorbance at 450 nm using a microplate reader (Corning Incorporated, Corning, NY, USA). Three replicated wells were used per experimental condition.

Annexin V/Propidium iodide staining. Cells were cultured at a 10^6 density and treated with corosolic acid and/or compound c for 24 h. Cells were centrifuged and washed three times with phosphate-buffered saline (PBS), and centrifuged. The supernatant was discarded and resuspended in 0.5 ml of cold PBS. The cells were processed and labeled according to the EzWay Annexin V-FITC Apoptosis Detection Kit that was used for this assay. The labeled cells were analyzed in a flow cytometer (BD FACSCanto™ II; BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting analysis. Cells were harvested using Trypsin-EDTA, washed twice with cold PBS, lysed with lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% NP-40, 1 mM PI, 1 mM DTT, 1 mM PMSF), and placed on ice for 1 h with occasional vortexing.

Centrifugation followed at 13,000 x g for 10 min at 4˚C to collect the supernatant. A Pierce BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. The cell lysate (50 µg) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 5% skim milk in PBS containing 0.05% Tween-20 for 1 h at 25˚C, then incubated with primary antibodies (1:1,000) overnight at 4˚C, followed by incubation with anti-rabbit horseradish peroxidase-conjugated IgG (1:3,000) for 2 h at room temperature and visualized with enhanced chemiluminescence.

Statistical analysis. All results presented were confirmed in at least three independent experiments. Data were presented as the mean ± standard deviation. Statistical differences were analyzed by one-way analysis of variance followed by Tukey's post hoc test using SPSS version 24.0 software (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of 5-FU resistant SNU-620 gastric cancer cells. We established a 5-FU-resistant gastric cancer cell line (SNU-620/5-FU^R) by continuous exposure of the parental cells SNU-620 to 7.5 µM 5-FU for approximately 6 months. TS protein level was markedly increased in SNU-620/5-FU^R cells compared to that in the 5-FU-sensitive parental cells based on Western blot analysis, indicating that SNU-620/5-FU^R cells were resistant to 5-FU (Fig. 1A and B). To confirm that SNU-620/5-FU^R cells were resistant to 5-FU, 5-FU was added at various concentrations (~750 µM). Cell viability was then determined by CCK-8 assay. 5-FU decreased cell viabilities of SNU-620 cells in a dose-dependent manner. However, it was not cytotoxic to SNU-620/5-FU^R cells (Fig. 1C).

AMPK phosphorylation level was reduced in 5-FU-resistant SNU-620/5-FU^R gastric cancer cells. AMPK phosphorylation was found to be reduced by 32.7% in 5-FU-resistant SNU-620/5-FU^R gastric cancer cells compared to the SNU-620 cells (Fig. 2A and B). To confirm the role of AMPK in cell resensitization to 5-FU, cells were treated with AMPK activator AICAR and the AMPK inhibitor compound c. TS protein expression and cell viability were then determined by western blotting analysis and CCK-8 assay. AICAR dramatically decreased TS expression (Fig. 2C and D) while AMPK inhibition by compound c treatment increased TS protein expression in SNU-620/5-FU^R cells (Fig. 2C and D). Activation of AMPK by AICAR decreased viability of SNU-620/5-FU^R cells. However, inhibition of AMPK by compound c did not decrease the viability of SNU-620/5-FU^R cells (Fig. 2E). These results suggest that 5-FU resistance is strongly regulated by AMPK. Therefore, AMPK phosphorylation might be a therapeutic target for overcoming 5-FU-resistance in gastric cancers.

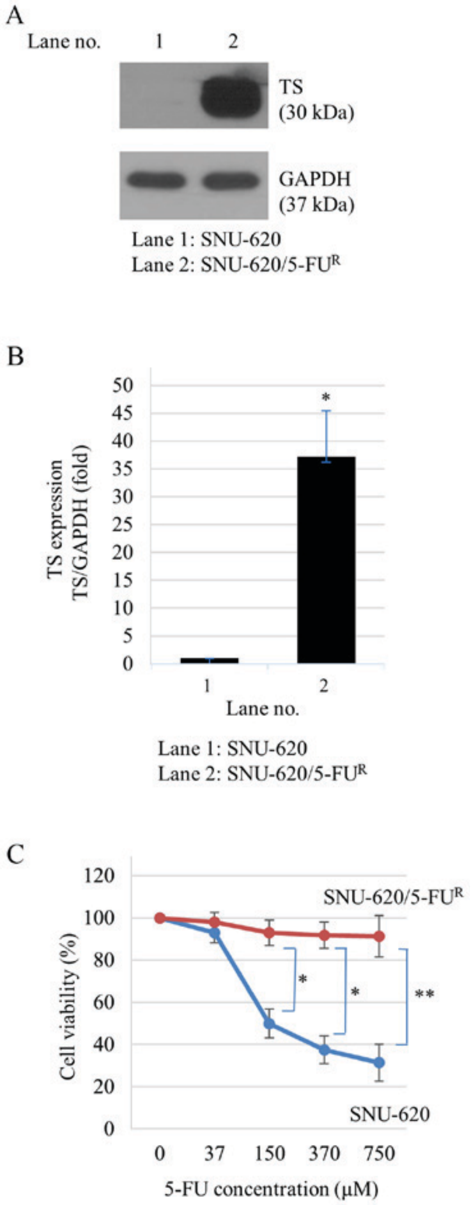

Figure 1. Establishment of 5-FU resistant SNU-620/5-FU^R human gastric cancer cells. (A) Protein expression level of TS was analyzed in 5-FU-sensitive SNU-620 and 5-FU-resistant SNU-620/5-FU^R cells. (B) Band intensities were normalized to the GAPDH expression level. $^*P<0.05$ vs. SNU-620 control (Lane no. 1). (C) SNU-620 and SNU-620/5-FU^R cells were cultured in the presence of 5-FU (0-750 µM) for 24 h and cell viability was determined by Cell Counting Kit-8 assay. Data are expressed as the mean ± standard deviation from three independent experiments. $^*P<0.05$ and $^{**}P<0.01$, as indicated. 5-FU, 5-fluorouracil; SNU-620/5-FU^R, 5-FU-resistant gastric cancer cell line; TS, thymidine synthase.

Corosolic acid activates AMPK and suppresses mTOR/4EBP1 phosphorylation in SNU-620/5-FU^R cells. Previous studies have reported that pharmacological activators of AMPK such as AICAR and metformin can induce apoptosis of gastric cancers (12,29). Moreover, corosolic acid, (2α,3β)-2,3-dihydroxyurs-12-en-28-oic acid (Fig. 3A) can activate AMPK and induce apoptosis in gastric cancers (25). Corosolic acid can also inhibit inflammation in adipose tissues (23). To evaluate the effect of corosolic acid on AMPK activation in SNU-620/5-FU^R cells, western blot analysis was performed. Results revealed that treatment with 10 and 25 µM corosolic acid dramatically increased AMPK phosphorylation (Fig. 3B). However, corosolic acid failed to activate AMPK in 5-FU sensitive SNU-620 gastric

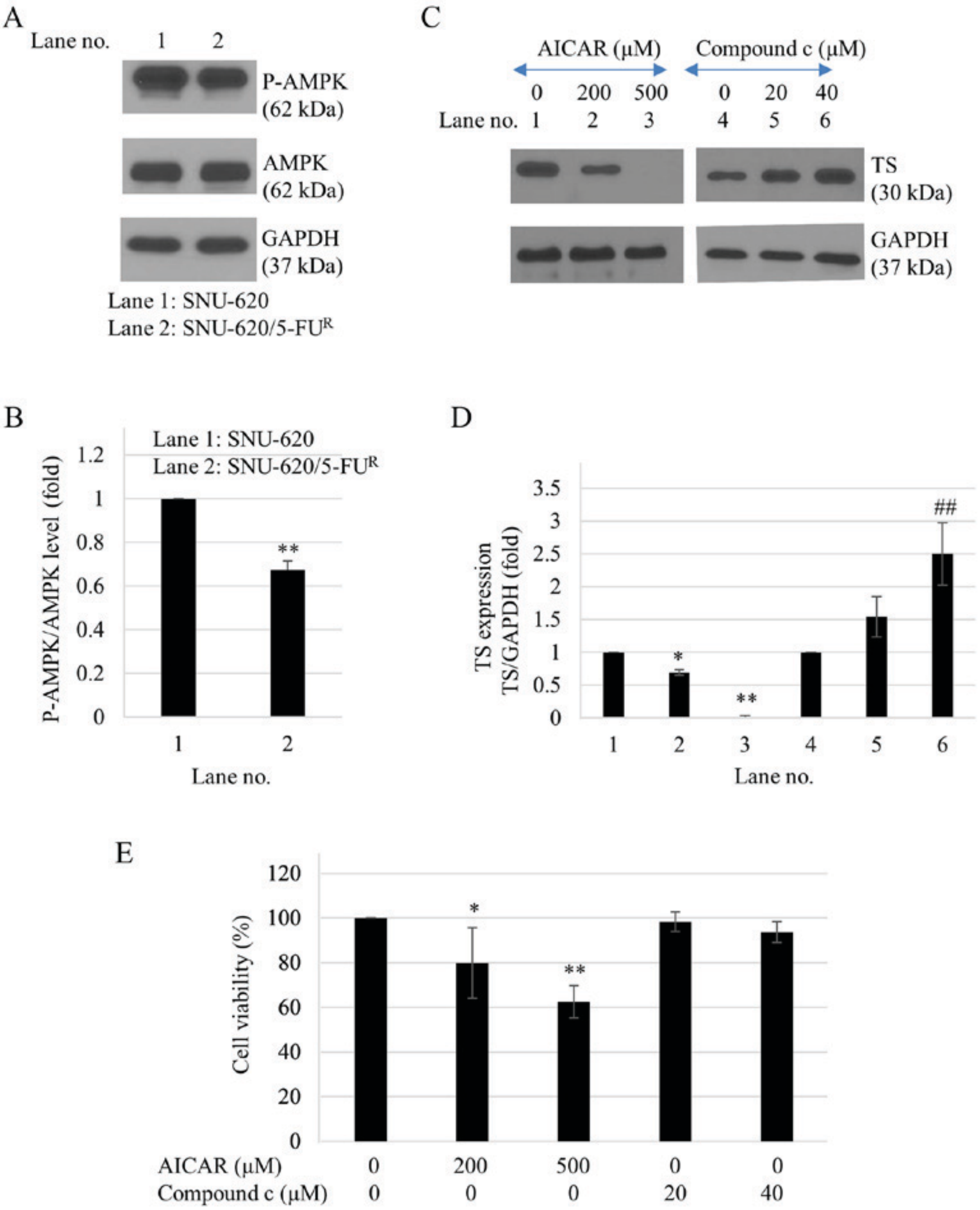

Figure 2. Reduced AMPK phosphorylation in 5-FU resistant gastric cancer cells. (A) AMPK phosphorylation in SNU-620 and SNU-620/5-FU^R cells was analyzed by western blot analysis. (B) Band intensities were normalized to those of AMPK. **P<0.01 vs. SNU-620 control (Lane no. 1). (C) Following 24 h treatment of SNU-620/5-FU^R cells with AICAR (200 and 500 μM) or compound c (20 and 40 μM), TS protein expression was determined by western blot analysis. (D) Band intensities were normalized to GAPDH level. (E) Following 24 h treatment of SNU-620/5-FU^R cells with AICAR (200 and 500 μM) or compound c (20 and 40 μM), cell viability was determined by Cell Counting Kit-8 assay. *P<0.05 and **P<0.01 vs. AICAR control (Lane no. 1); $^{\#\#}$P<0.01 vs. Compound c control (Lane no. 4). AMPK, adenosine monophosphate-activated protein kinase; 5-FU, 5-fluorouracil; SNU-620/5-FU^R, 5-FU-resistant gastric cancer cell line; TS, thymidine synthase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide.

cancer cells (data not shown). We also tested the status of mTOR/4EBP1, a downstream molecular marker of AMPK signaling. Activated AMPK inhibited the activation of mTOR/4EBP1 in corosolic acid treated SNU-620/5-FU^R cells (Fig. 3B). Corosolic acid decreased the level of TS expression in a dose-dependent manner, with a pattern similar to that of AMPK activation (Fig. 3B). This suggests possible cross-talk between AMPK and 5-FU resistance after corosolic acid treatment. To determine whether corosolic acid-induced AMPK activation was associated with enhanced growth rate of SNU-620/5-FU^R cells after treatment with compound c (40 μM) and/or corosolic acid (1, 10, 25 and 50 μM), cell viability was measured by CCK-8 assay. Results are shown in Fig. 3C. Compared to solo treatment with 10 or 25 μM corosolic acid, additional compound c treatment increased cell viabilities by 27.1 and 40%, respectively. These results suggest that corosolic acid-induced AMPK activation might be a mechanism involved in 5-FU resistance.

Corosolic acid resensitizes SNU-620/5-FU^R gastric cancer cells to 5-FU. To investigate the sensitization effect of corosolic acid on 5-FU-resistant gastric cancer cells, SNU-620/5-FU^R cells were treated with 5-FU (150 μM, 50% inhibitory concentration in 5-FU sensitive SNU-620 cells) and/or corosolic acid and growth rates were measured by CCK-8 assay. Results showed that single treatment with 150 μM of 5-FU or 25 μM of

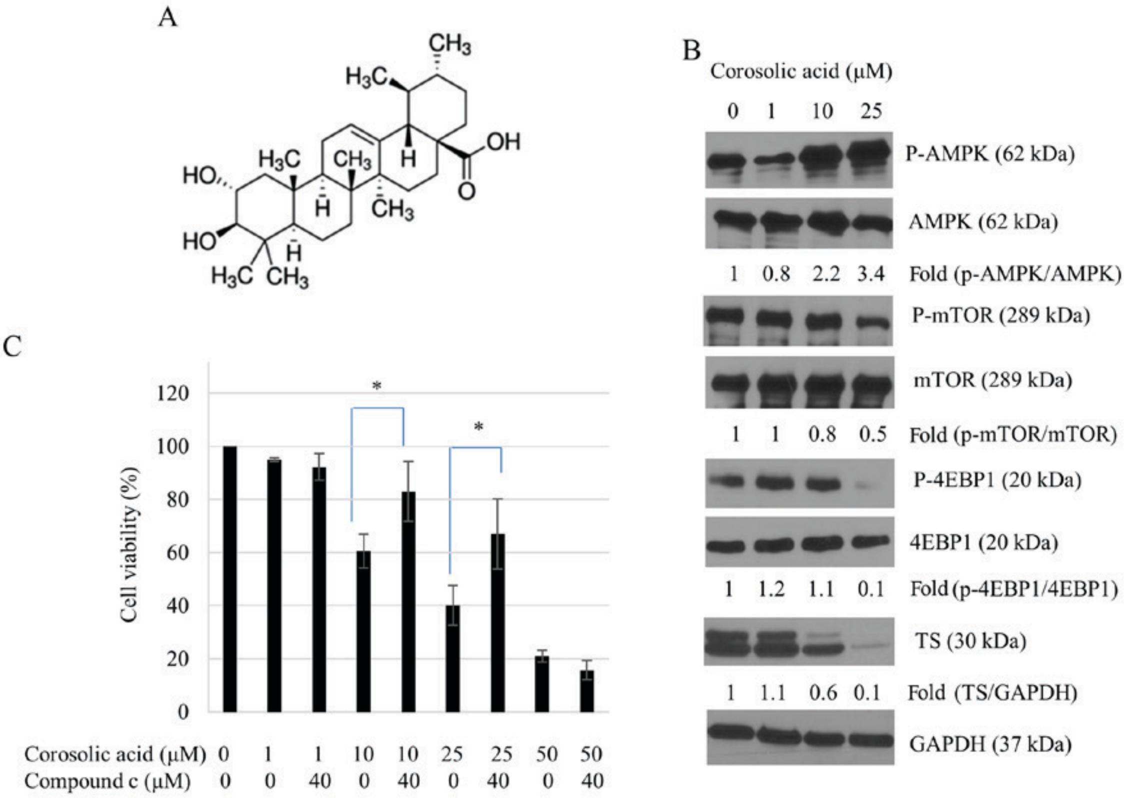

Figure 3. Corosolic acid activates AMPK signaling in SNU-620/5-FU^R cells. (A) Structure of corosolic acid [(2α,3β)-2,3-Dihydroxyurs-12-en-28-oic acid, 2α-Hydroxyursolic acid]. (B) Cells were incubated with corosolic acid for 24 h and equal amounts of protein samples were resolved by SDS-PAGE followed by immunoblotting using antibodies against AMPK, p-AMPK, mTOR, p-mTOR, 4EBP1, and P-4EBP1. GAPDH expression served as an internal control. (C) Cell growth inhibition was determined by Cell Counting Kit-8 assay. Values are presented as the mean ± standard deviation of three independent experiments. $^*P<0.05$, as indicated. AMPK, adenosine monophosphate-activated protein kinase; 5-FU, 5-fluorouracil; SNU-620/5-FU^R, 5-FU-resistant gastric cancer cell line; TS, thymidine synthase; p-, phosphorylated; mTOR, mammalian target of rapamycin; 4EBP1, 4E-binding protein 1.

corosolic acid decreased the growth rate SNU-620/5-FU^R cells by 93.4 and 42.7%, respectively. However, combination treatment significantly enhanced sensitivity of SNU-620/5-FU^R cells to 5-FU (Fig. 4A). To estimate the sensitization effect of corosolic acid, 5-FU-sensitive cells were also exposed to 5-FU (150 μM) with or without corosolic acid. However, the combination effect observed in SNU-620/5-FU^R cells was not evident in SNU-620 cells (Fig. 4A). In addition, TS expression in SNU-620/5-FU^R cells was diminished by treatment with corosolic acid alone. It was drastically reduced following treatment with corosolic acid and 5-FU in combination (Fig. 4B and C). These results suggest that corosolic acid can probably sensitize 5-FU resistance.

Corosolic acid enhances sensitivity of SNU-620/5-FU^R gastric cancer cells to 5-FU by upregulating AMPK. The effect of corosolic acid on AMPK activation in the presence or absence of 5-FU was tested in SNU-620/5-FU^R cells. 5-FU (150 μM) moderately increased AMPK activation. A combination of corosolic acid (25 μM) with 5-FU was more effective. However, compound c significantly blocked AMPK phosphorylation (Fig. 5A and B). Compared to treatment with corosolic acid or 5-FU alone, the combination of corosolic acid and 5-FU resulted in increased caspase-3 and PARP cleavage and cytochrome c translocation, while compound c partly blocked 5-FU+corosolic acid-induced apoptotic activities (Fig. 5C). Apoptotic cell percentages were examined after treated with corosolic acid, 5-FU, or compound c, by flow cytometric analysis. A significant increase in the percentage of apoptotic cells was observed in SNU-620/5-FU^R gastric cancer cells after treatment with corosolic acid in the presence of 5-FU. However, compound c significantly blocked apoptosis compared to a combination of corosolic acid and 5-FU (Fig. 5D). Percentages of early and late apoptotic/necrotic cells were increased from 0.5±0.1 to 21.7±2.0% in corosolic acid (25 μM) treated cells, 68.6±13.7% in cells treated with a combination of corosolic acid (25 μM) and 5-FU (150 μM), and 39.7±2.8% in cells treated with combination of corosolic acid (25 μM), 5-FU (150 μM), and compound c (40 μM) (Fig. 5E). Compound c significantly decreased the percentage of apoptotic cells compared to corosolic acid or 5-FU (Fig. 5D and E). These results suggest that corosolic acid-induced AMPK activation plays a key role in enhancing sensitivity of 5-FU-resistant gastric cancer cells to 5-FU.

Discussion

5-FU is a heterocyclic aromatic organic compound with a structure similar to that of pyrimidine molecules of DNA and RNA. It is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen (3). 5-FU is used for treating gastric cancer. It can increase overall survival by 6% and reduce the risk of mortality by 18% (30). However, the occurrence of resistance to 5-FU treatment is a major

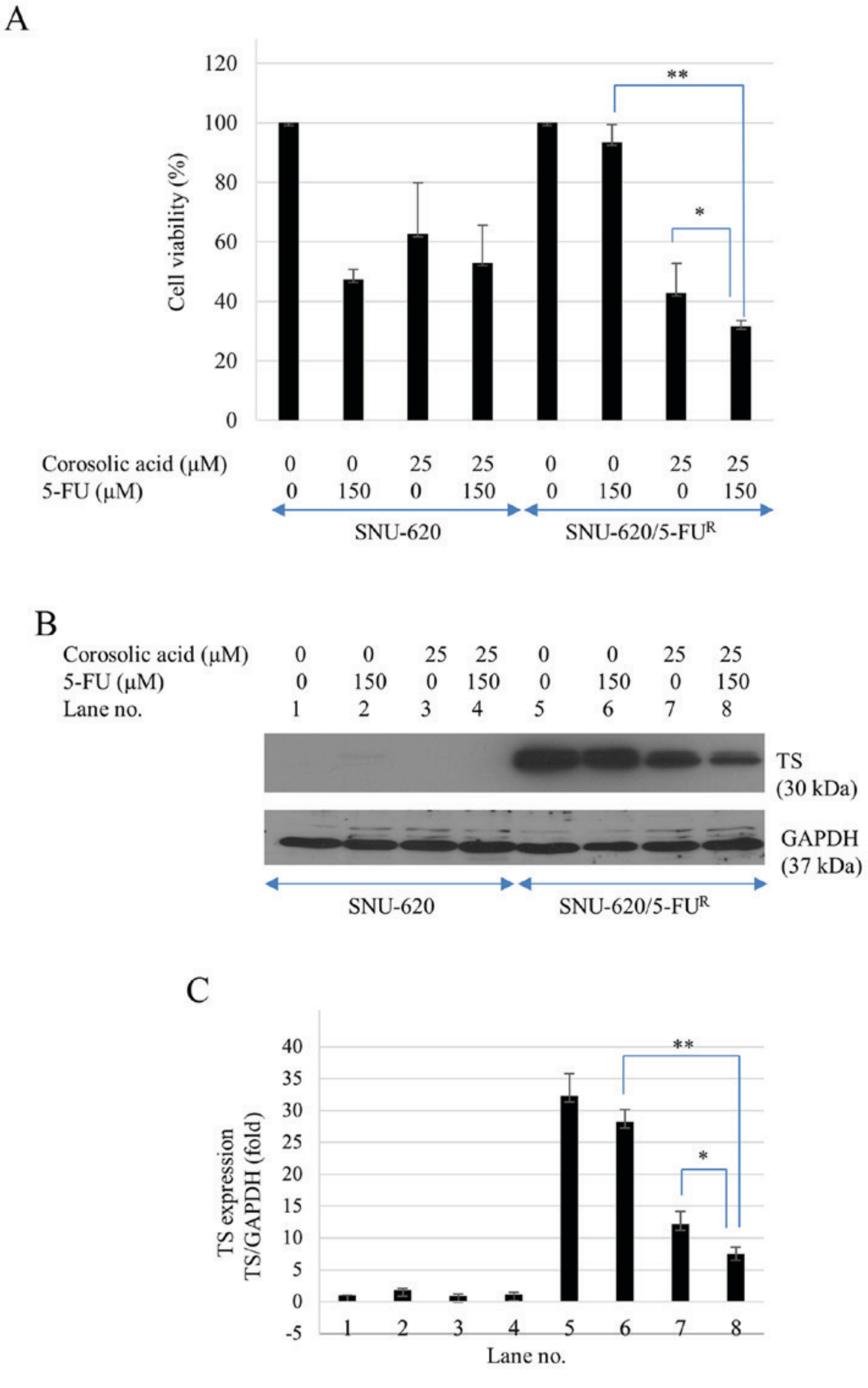

Figure 4. Corosolic acid sensitizes 5-FU-resistant SNU-620/5-FU^{R} gastric cancer cells. (A) Cell viability of SNU-620/5-FU^{R} and SNU-620 cells treated with 5-FU with or without corosolic acid for 24 h based on Cell Counting Kit-8 assay. Values are presented as the mean ± standard deviation of three independent experiments. (B) SNU-620 and SNU-620/5-FU^{R} cells were treated with corosolic acid and 5-FU at the indicated concentrations for 24 h. TS protein expression was determined by western blot analysis. GAPDH expression was used as an internal control. (C) Band intensities were normalized to GAPDH level. *P<0.05 and **P<0.01, as indicated. 5-FU, 5-fluorouracil; SNU-620/5-FU^{R}, 5-FU-resistant gastric cancer cell line; TS, thymidine synthase.

problem for most gastric cancer patients, resulting in limited overall efficacy (31). High-level expression of TS, increased activity of deoxyuridine triphosphatase, methylation of MLH1 gene, and overexpression of Bcl-2, Bcl-XL, and Mcl-1 proteins have been reported to be associated with cancer resistance to 5-FU (3). Although the precise mechanism involved in gastric cancer resistance to 5-FU remains unknown, several reports have suggested that AMPK might be a biological predictor and beneficial target for cancer treatment through metabolic alteration (32). Previous clinical studies have shown that increases in phosphorylated AMPK is associated with tumor grade and prognosis for several solid tumors (33). More recently, phosphorylated AMPK levels have been reported as being significantly reduced in 5-FU-resistant gastric cancer cells (AGS cells) compared to 5-FU-sensitive cells (SGC-7901 cells) (12). In this study,

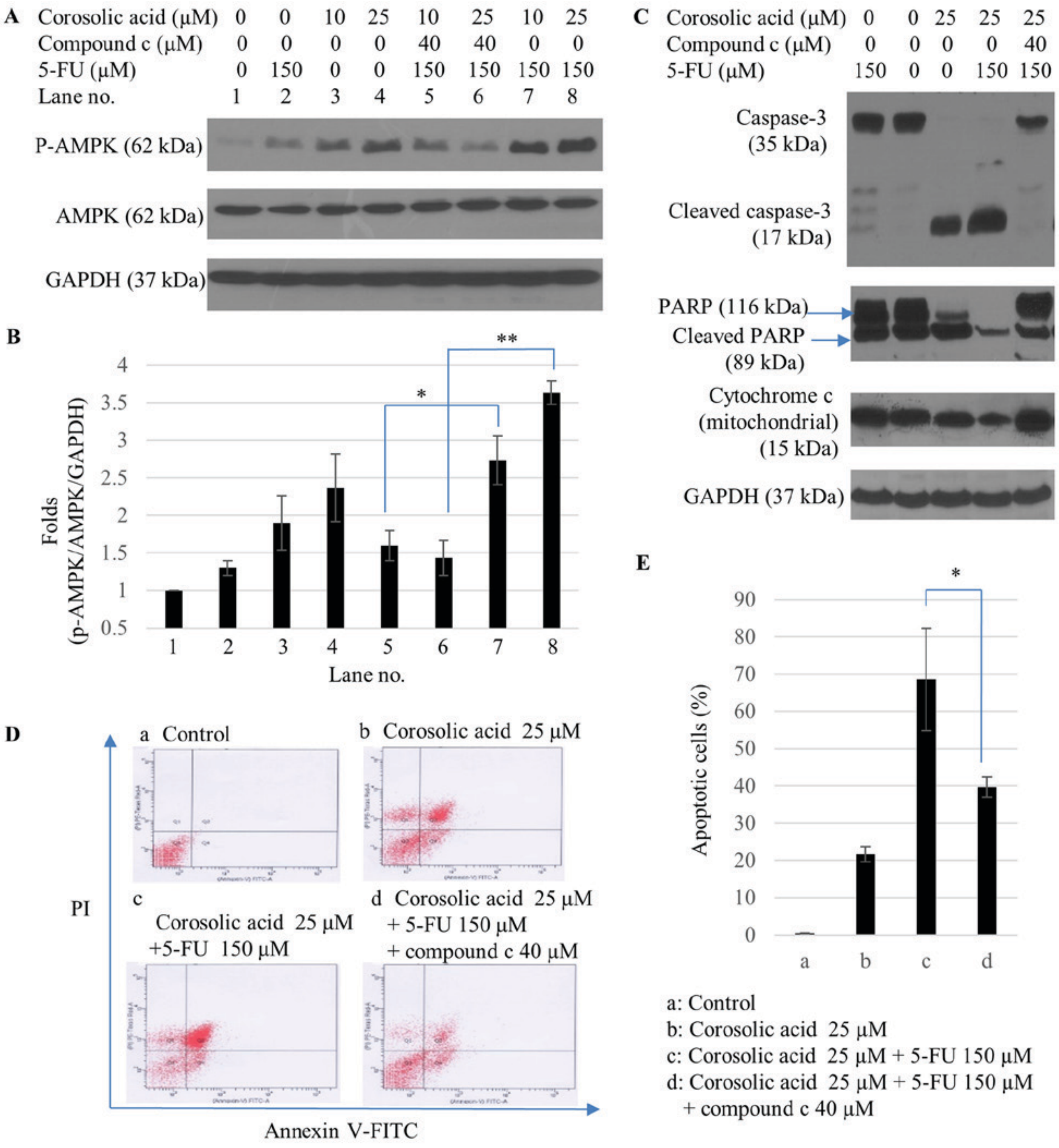

Figure 5. Corosolic acid-induced AMPK activation enhances 5-FU sensitivity in SNU-620/5-FU^{R} gastric cancer cells (A) Following treatment with corosolic acid (10 and 25 µM), 5-FU (150 µM), or compound c (40 µM), AMPK and p-AMPK protein expression levels were determined by western blot analysis. (B) Values are presented as the mean ± standard deviation of three independent experiments. (C) Activation of apoptosis in cells treated with 5-FU (150 µM), corosolic acid (25 µM), or compound c (40 µM) was measured by caspase-3 and PARP cleavages and cytochrome c located in mitochondria based on western blot analysis. GAPDH was used as internal protein loading control. (D) Apoptotic cell population was evaluated by flow cytometry analysis following double staining with Annexin V and PI. (E) Percentages of early apoptosis plus late apoptosis/necrosis are shown in the bar graph. *P<0.05 and **P<0.01, as indicated. AMPK, adenosine monophosphate-activated protein kinase; 5-FU, 5-fluorouracil; SNU-620/5-FU^{R}, 5-FU-resistant gastric cancer cell line; TS, thymidine synthase; p-, phosphorylated; PARP, poly-(adenosine diphosphate-ribose) polymerase; PI, propidium iodide; FITC, fluorescein isothiocyanate.

we demonstrated that AMPK phosphorylation level was significantly decreased in 5-FU resistant gastric cancer cells (SNU-620/5-FU^{R}) compared to sensitive gastric cancer cells (SNU-620). The present study aimed to identify alterative therapeutic approaches for enhancing 5-FU sensitivity by activating AMPK. TS expression level was decreased by AICAR, an AMPK activator. Consistent with this finding, treatment with the AMPK inhibitor, compound c, increased TS expression. AICAR significantly decreased viability of 5-FU resistant cells without altering viability of 5-FU sensitive cells (data not shown). Even though compound c is widely known as an AMPK inhibitor, this compound is involved in killing cancer cells by multiple mechanisms (Calpain/Cathepsin pathway; AKT; mTORC1/C2; cell cycle block; necroptosis; autophagy) (34). Liu *et al* (34) suggests that compound c kills cancer cells by an AMPK-independent mechanism. Therefore, we need to perform further research to find out why compound c did not increase cell viability in gastric cancer cells. These data suggest that AMPK might be a potent regulator of 5-FU resistance in gastric cancers.

Several studies have demonstrated that corosolic acid can activate AMPK in adipose tissue, endothelial cells, and gastric cancer cells (23-25). A recent clinical study revealed that metformin, an AMPK activator, can reduce gastric cancer risk in patients with type 2 diabetes mellitus (35). In addition, metformin can reverse multidrug resistance in breast cancer cells and hepatocellular carcinoma Bel-7402/5-fluorourscil cells (10,11). In the present study, we found that corosolic acid activated AMPK phosphorylation in 5-FU resistant SNU-620/5-FU^R cells followed by decreased phosphorylation levels of mTOR/4EBP1. Corosolic acid-induced AMPK activation down-regulated cell viability in a dose-dependent manner. However, AMPK activity was significantly inhibited by compound c in 5-FU resistant SNU-620/5-FU^R cells. Therefore, corosolic acid-induced AMPK activation plays an important role in overcoming 5-FU-resistance in gastric cancer. In Fig. 3C, 1 μM corosolic acid + compound c (Lane 3) and 50 μM corosolic acid + compound c (Lane 9) did not preserve cell viability. 1 μM corosolic acid and 50 μM corosolic acid did not activate AMPK in our experiments, which might be a possible reason corosolic acid-treated cells could not be reversed by compound c. To investigate the sensitization effect of corosolic acid to 5-FU, resistant or sensitive cells were treated with corosolic acid (25 μM) in the presence or absence of 5-FU (150 μM). Viability of SNU-620/5-FU^R cells treated with 5-FU in the presence of corosolic acid was significantly inhibited. However, no difference in viability of SNU-620 cells was found after such treatment. Corosolic acid in combination with 5-FU significantly decreased TS expression level in SNU-620/5-FU^R cells compared to treatment with corosolic acid or 5-FU alone. Because TS is a major marker of 5-FU resistance status, these results indicate that corosolic acid might be able to reverse and/or sensitize 5-FU resistance of SNU-620/5-FU^R cells.

AMPK activation is known to play an important role in enhancing chemosensitivity to certain chemotherapeutic agents such as 5-FU in breast cancer, intrahepatic cholangiocarcinoma, and gall bladder cancer (10,11,36). To investigate corosolic acid-induced AMPK activation involved in the resensitization effect of 5-FU, we determined AMPK phosphorylation level in the presence or absence of 5-FU. Our results showed that corosolic acid (10 and 25 μM) synergistically enhanced p-AMPK in the presence of 5-FU. Sensitivity of cells to chemotherapy might be estimated by examining apoptosis. The apoptotic rate was analyzed by western blot to detect caspase-3 and PARP cleavage, as well as cytochrome c translocation, and Annexin V-PI staining. The expression of cleaved caspase-3 and PARP and cytochrome c translocation in SNU-620/5-FU^R cells were increased by 5-FU. The apoptotic rate in SNU-620/5-FU^R cells treated with a combination of corosolic acid and 5-FU was significantly higher than when treated with corosolic acid alone. Chemically inhibited AMPK activation by compound c abolished AMPK phosphorylation and apoptotic activities in the presence of 5-FU. These findings suggest that corosolic acid might be able to enhance chemosensitivity of gastric cancer to 5-FU.

We agree with that gastric cancer is a heterogenous disease. To investigate the effect of corosolic acid on 5-FU chemoresistance in various gastric cancer cell types, several gastric cancer cell lines (SNU-1, SNU-5, AGS, SNU-484, SNU-601, and NCI-N87), have being established that are 5-FU resistant. Investigations of these are planned.

In conclusion, our study revealed that 5-FU resistant gastric cancer cells (SNU-620/5-FU^R) had lower phosphorylated AMPK expression than 5-FU sensitive parental cells (SNU-620). Therefore, AMPK expression might be a possible treatment target for 5-FU resistant gastric cancers. We also found that corosolic acid could sensitize 5-FU resistance and inhibit viability of 5-FU resistant gastric cancer cells by activating the AMPK pathway. Therefore, corosolic acid could be used as an effective complimentary medicine to restore chemosensitivity of drug resistant gastric cancer cells to 5-FU. Further studies need to focus on chemotherapeutic sensitization by corosolic acid in combination with other chemotherapeutics, and to investigate the detailed molecular mechanisms involved.

Acknowledgements

The present study is based on a doctoral thesis (Chungnam National University, 2017).

Funding

The present study was supported by the Research Fund of Chungnam National University (grant no. 2015109701).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JBP designed the study and prepared the manuscript. JSL contributed to the conception of the study, analyzed the data and drafted the manuscript. MSL, EYC and SK performed the experiments. JYS was involved in the study conception and design, and revised the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Smith JK, McPhee JT, Hill JS, Whalen GF, Sullivan ME, Litwin DE, Anderson FA and Tseng JF: National outcomes after gastric resection for neoplasm. Arch Surg 142: 387-393, 2007.

2. Tsai MM, Wang CS, Tsai CY, Chi HC, Tseng YH and Lin KH: Potential prognostic, diagnostic and therapeutic markers for human gastric cancer. World J Gastroenterol 20: 13791-13803, 2014.
3. Zhang N, Yin Y, Xu SJ and Chen WS: 5-Fluorouracil: Mechanisms of resistance and reversal strategies. Molecules 13: 1551-1569, 2008.
4. Scartozzi M, Maccaroni E, Giampieri R, Pistelli M, Bittoni A, Del Prete M, Berardi R and Cascinu S: 5-Fluorouracil pharmacogenomics: Still rocking after all these years? Pharmacogenomics 12: 251-265, 2011.
5. Johnston PG, Lenz HJ, Leichman CG, Danenberg KD, Allegra CJ, Danenberg PV and Leichman L: Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. Cancer Res 55: 1407-1412, 1995.
6. Saga Y, Suzuki M, Mizukami H, Urabe M, Fukushima M, Ozawa K and Sato I: Enhanced expression of thymidylate synthase mediates resistance of uterine cervical cancer cells to radiation. Oncology 63: 185-191, 2002.
7. Copur S, Aiba K, Drake JC, Allegra CJ and Chu E: Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. Biochem Pharmacol 49: 1419-1426, 1995.
8. Fakhrejahani E, Miyamoto A and Tanigawa N: Correlation between thymidylate synthase and dihydropyrimidine dehydrogenase mRNA level and in vitro chemosensitivity to 5-fluorouracil, in relation to differentiation in gastric cancer. Cancer Chemother Pharmacol 60: 437-446, 2007.
9. Kim YH, Liang H, Liu X, Lee JS, Cho JY, Cheong JH, Kim H, Li M, Downey TJ, Dyer MD, *et al*: AMPKα modulation in cancer progression: Multilayer integrative analysis of the whole transcriptome in Asian gastric cancer. Cancer Res 72: 2512-2521, 2012.
10. Ling S, Tian Y, Zhang H, Jia K, Feng T, Sun D, Gao Z, Xu F, Hou Z, Li Y and Wang L: Metformin reverses multidrug resistance in human hepatocellular carcinoma Bel-7402/5-fluorouracil cells. Mol Med Rep 10: 2891-2897, 2014.
11. Qu C, Zhang W, Zheng G, Zhang Z, Yin J and He Z: Metformin reverses multidrug resistance and epithelial-mesenchymal transition (EMT) via activating AMP-activated protein kinase (AMPK) in human breast cancer cells. Mol Cell Biochem 386: 63-71, 2014.
12. Wu Y, Qi Y, Liu H, Wang X, Zhu H and Wang Z: AMPK activator AICAR promotes 5-FU-induced apoptosis in gastric cancer cells. Mol Cell Biochem 411: 299-305, 2016.
13. Hua HW, Jiang F, Huang Q, Liao ZJ and Ding G: Re-sensitization of 5-FU resistance by SPARC through negative regulation of glucose metabolism in hepatocellular carcinoma. Tumour Biol 36: 303-313, 2015.
14. Hou W, Li Y, Zhang Q, Wei X, Peng A, Chen L and Wei Y: Triterpene acids isolated from *Lagerstroemia speciosa* leaves as alpha-glucosidase inhibitors. Phytother Res 23: 614-618, 2009.
15. Thuong PT, Min BS, Jin W, Na M, Lee J, Seong R, Lee YM, Song K, Seong Y, Lee HK, *et al*: Anti-complementary activity of ursane-type triterpenoids from *Weigela subsessilis*. Biol Pharm Bull 29: 830-833, 2006.
16. Fukushima M, Matsuyama F, Ueda N, Egawa K, Takemoto J, Kajimoto Y, Yonaha N, Miura T, Kaneko T, Nishi Y, *et al*: Effect of corosolic acid on postchallenge plasma glucose levels. Diabetes Res Clin Pract 73: 174-177, 2006.
17. Miura T, Ueda N, Yamada K, Fukushima M, Ishida T, Kaneko T, Matsuyama F and Seino Y: Antidiabetic effects of corosolic acid in KK-Ay diabetic mice. Biol Pharm Bull 29: 585-587, 2006.
18. Xu Y, Zhao Y, Xu Y, Guan Y, Zhang X, Chen Y, Wu Q, Zhu G, Chen Y, Sun F, *et al*: Blocking inhibition to YAP by ActinomycinD enhances anti-tumor efficacy of Corosolic acid in treating liver cancer. Cell Signal 29: 209-217, 2017.
19. Sung B, Kang YJ, Kim DH, Hwang SY, Lee Y, Kim M, Yoon JH, Kim CM, Chung HY and Kim ND: Corosolic acid induces apoptotic cell death in HCT116 human colon cancer cells through a caspase-dependent pathway. Int J Mol Med 33: 943-949, 2014.
20. Nho KJ, Chun JM and Kim HK: Corosolic acid induces apoptotic cell death in human lung adenocarcinoma A549 cells in vitro. Food Chem Toxicol 56: 8-17, 2013.
21. Lee HS, Park JB, Lee MS, Cha EY, Kim JY and Sul JY: Corosolic acid enhances 5-fluorouracil-induced apoptosis against SNU-620 human gastric carcinoma cells by inhibition of mammalian target of rapamycin. Mol Med Rep 12: 4782-4788, 2015.
22. Lee MS, Cha EY, Thuong PT, Kim JY, Ahn MS and Sul JY: Down-regulation of human epidermal growth factor receptor 2/neu oncogene by corosolic acid induces cell cycle arrest and apoptosis in NCI-N87 human gastric cancer cells. Biol Pharm Bull 33: 931-937, 2010.
23. Yang J, Leng J, Li JJ, Tang JF, Li Y, Liu BL and Wen XD: Corosolic acid inhibits adipose tissue inflammation and ameliorates insulin resistance via AMPK activation in high-fat fed mice. Phytomedicine 23: 181-190, 2016.
24. Li Y, Zhou ZH, Chen MH, Yang J, Leng J, Cao GS, Xin GZ, Liu LF, Kou JP, Liu BL, *et al*: Inhibition of mitochondrial fission and NOX2 expression prevent NLRP3 inflammasome activation in the endothelium: The role of corosolic acid action in the amelioration of endothelial dysfunction. Antioxid Redox Signal 24: 893-908, 2016.
25. Lee MS, Lee CM, Cha EY, Thuong PT, Bae K, Song IS, Noh SM and Sul JY: Activation of AMP-activated protein kinase on human gastric cancer cells by apoptosis induced by corosolic acid isolated from *Weigela subsessilis*. Phytother Res 24: 1857-1861, 2010.
26. Goel A and Aggarwal BB: Curcumin, the golden spice from Indian saffron, is a chemosensitizer and radiosensitizer for tumors and chemoprotector and radioprotector for normal organs. Nutr Cancer 62: 919-930, 2010.
27. Tang H, Zhang X, Cui S, Wang J, Ruan Q, Huang Y and Yang D: Role and mechanism research on reversal of 5-fluorouracil resistance by epigallocatechin gallate in gastric cancer drug-resistance cells lines SGC-7901/5-FU. Zhonghua Wei Chang Wai Ke Za Zhi 19: 1170-1175, 2016 (In Chinese).
28. Liao F, Yang Z, Lu X, Guo X and Dong W: Sinomenine sensitizes gastric cancer cells to 5-fluorouracil in vitro and in vivo. Oncol Lett 6: 1604-1610, 2013.
29. Kato K, Gong J, Iwama H, Kitanaka A, Tani J, Miyoshi H, Nomura K, Mimura S, Kobayashi M, Aritomo Y, *et al*: The antidiabetic drug metformin inhibits gastric cancer cell proliferation in vitro and in vivo. Mol Cancer Ther 11: 549-560, 2012.
30. Kim DC, Park KR, Jeong YJ, Yoon H, Ahn MJ, Rho GJ, Lee J, Gong YD and Han SY: Resistance to the c-Met inhibitor KRC-108 induces the epithelial transition of gastric cancer cells. Oncol Lett 11: 991-997, 2016.
31. Holohan C, Van Schaeybroeck S, Longley DB and Johnston PG: Cancer drug resistance: An evolving paradigm. Nat Rev Cancer 13: 714-726, 2013.
32. Wang W and Guan KL: AMP-activated protein kinase and cancer. Acta Physiol (Oxf) 196: 55-63, 2009.
33. Hadad SM, Baker L, Quinlan PR, Robertson KE, Bray SE, Thomson G, Kellock D, Jordan LB, Purdie CA, Hardie DG, *et al*: Histological evaluation of AMPK signaling in primary breast cancer. BMC Cancer 9: 307, 2009.
34. Liu X, Chhipa RR, Nakano I and Dasgupta B: The AMPK inhibitor compound C is a potent AMPK-independent antiglioma agent. Mol Cancer Ther 13: 596-605, 2014.
35. Tseng CH: Metformin reduces gastric cancer risk in patients with type 2 diabetes mellitus. Aging (Albany NY) 8: 1636-1649, 2016.
36. Gao H, Xie J, Peng J, Han Y, Jiang Q, Han M and Wang C: Hispidulin inhibits proliferation and enhances chemosensitivity of gallbladder cancer cells by targeting HIF-1α. Exp Cell Res 332: 236-246, 2015.