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Protective Autophagy in 5-Fluorouracil-Resistant Colorectal Cancer Cells and ERK-RSK-ABCG2 Linkage

Autofagia Protectora en Células de Cáncer Colorrectal Resistentes al 5-Fluorouracilo y Enlace ERK-RSK-ABCG2

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SUMMARY: To evaluate the anti-cancer effects of yeast extract on resistant cells, autophagy and necroptosis were investigated in 5-fluorouracil (5-FU)-resistant colorectal cancer cells. Further underlying characteristics on drug resistance were evaluated, focused on ERK-RSK-ABCG2 linkage. SNU-C5 and 5-FU resistant SNU-C5 (SNU-C5/5-FUR) colorectal cancer cells were adopted for cell viability assay and Western blotting to examine the anti-cancer effects of yeast extract. Yeast extract induced autophagy in SNU-C5 cells with increased Atg7, Atg12-5 complex, Atg16L1, and LC3 activation (LC3-II/LC3-I), but little effects in SNU-C5/5-FUR cells with increased Atg12-5 complex and Atg16L1. Both colorectal cancer cells did not show necroptosis after yeast extract treatment. Based on increased ABCG2 and RSK expression after yeast extract treatment, drug resistance mechanisms were further evaluated. As compared to wild type, SNU-C5/5-FUR cells showed more ABCG2 expression, less RSK expression, and less phosphorylation of ERK. ABCG2 inhibitor, Ko143, treatment induces following changes: 1) more sensitivity at 500 mM 5-FU, 2) augmented proliferation, and 3) less phosphorylation of ERK. These results suggest that protective autophagy in SNU-C5/5-FUR cells with increased ABCG2 expression might be candidate mechanisms for drug resistance. As the ERK responses were different from each stimulus, the feasible mechanisms among ERK-RSK-ABCG2 should be further investigated in 5-FU-resistant CRC cells.

KEYWORDS: Autophagy; Colorectal cancer; Drug Resistance; ERK; 5-fluorouracil.

INTRODUCTION

Colorectal cancer (CRC) is the third-most common cancer diagnosis and the third leading cause of cancer death worldwide (Cancer Org., 2023) and also in South Korea (KOSIS, 2023). Although the rate of people being diagnosed with CRC each year has dropped, mortality rates show opposite results in the US and South Korea. The death rate from CRC in the US has dropped in both men and women due to improved screening, which results in many CRCs being found earlier and easier to treat (Cancer Org., 2023). The death rate from CRC, however, has increased for the last decade due to aging in South Korea (KOSIS, 2023).

Yeast extract (YE) has been known to show contradictory effects on antimitotic activity of 5-fluorouracil (5-FU) depend on cells, but does not interfere with the effects in vivo (Cook *et al*., 1975). We found incidentally that YE has anti-proliferative effects on renal cell carcinoma cells as compared with normal kidney proximal tubule cells (HK-2 cell line) (Moon *et al*., 2019). The highest concentration of YE, which did not affect the survival rate of HK-2 cells, induced cell cycle arrest at G0/G1 phase while it did not affect cell death pathways including apoptosis, necrosis, and autophagy. The anti-proliferative effects of YE were further examined in CRC cells, wild type and 5-FU-resistant SNU-C5 cell lines (Moon *et al.,* 2020). Regardless of 5-FU-resistance, YE induced cell cycle arrest at G0/G1 phase with increased p21 and decreased free iron levels as expected. Contrary to the renal cell carcinoma cells, YE induced apoptosis in both CRC cells through the increased phosphorylation of p38 and p53. The YE-induced apoptosis was blocked by a PARP inhibitor (3-aminobenzmide). Therefore, we suggested that YE inhibited the proliferation of CRC cells and induced apoptosis via the activation of the p38-p53-p21 cascade.

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There are a number of cell deaths, in which some share a resemblance to apoptosis and or necrosis (Nirmala & Lopus, 2020). Apoptosis and autophagy has been proposed as programmed cell death type I and type II, respectively (Li *et al*., 2020). Autophagy is a sequential process of catabolism of dysfunctional proteins and can be enhanced in response to stresses to preserve homeostasis (Li *et al*., 2017). Autophagy is regulated by a series of autophagy-related genes (Atg) and the microtubule-associated protein 1 light chain 3 (LC3) (Li *et al*., 2017, 2020). To form LC3-II, LC3-I should be first conjugated with Atg3 and Atg7, and then along with Atg12-Atg5-Atg16 complex. Since LC3-II is attached to the autophagosome membranes, it is widely used as a marker for assessing autophagy (Schaaf *et al*., 2016; Lim *et al*., 2021). Necroptosis shares some standard features with necrosis. Necroptosis is negatively regulated by caspases and initiated by a complex containing of receptor-interacting serine/threonine kinase (RIP) and RIP3 kinases, leading to phosphorylation of mixed lineage kinase domain like protein (MLKL) (Nirmala & Lopus, 2020).

While the main pathway was apoptosis, different auxiliary pathways were suggested depend on 5-FU resistance (Park *et al*., 2022), I focused on the fact of no difference on cell death responses between the wild type and 5-FU-resistant CRC cells under YE treatment with the opposite results on extracellular signal-regulated protein kinase (ERK) (Moon *et al*., 2020). Recently, various mechanisms of resistance to 5-FU are summarized including decreased apoptosis, increased protective autophagy, and increased drug efflux via ATP-binding cassette (ABC) sub-family G member 2 (ABCG2) (Azwar *et al*., 2021). Therefore, the aim was to investigate which cell death pathway including autophagy was specific to 5-FU-resistant CRC cells as compared with wild type cells under YE treatment. Accordingly, the feasible mechanisms through ERK or ABCG2 were examined in both CRC cells, and the differences between wild type and 5-FU-resistant CRC cells were revealed.

MATERIAL AND METHOD

Reagents and antibodies. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Amresco, Inc. (VWR International LLC, Seongnam, Republic of Korea). 5-FU (#F6627), and YE (#Y1625) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Ko143 (#3241), a selective ABCG2 inhibitor (known EC90 = 26 nM), was purchased from Tocris Bioscience (Bio-Techne Korea, Gyeonggi-Do, Republic of Korea).

The antibodies specific for ERK (1:1,000; #sc-93) and glyceraldehyde 3-phophage dehydrogenase (GAPDH; 1:2,000; #sc-47724) were obtained from Santa Cruz Biotechnology (Dallas, TX); autophagy antibody sampler kit (1:1,000/each; #4445; consisting of LC3-I/II (#12741), Atg5 (#12994), Atg12 (#4180), Atg16L1 (#8089), Atg7 (#8558) and Atg3 (#3415)), Caspase-8 (1:1,000, #9746), necroptosis antibody sampler kit (1:1,000/each; #98110) consisting of RIP (#3493), phosphor-RIP (#65746), MLKL (#14993), phosphor-MLKL (#91689), RIP3 (#13526) and phosphor-RIP3 (#93654)) and Phospho-ERK (1:1,000; #4370) were obtained from Cell Signaling Technology (Danvers, MA); ABCG2 (1:500; #MAB4155, Millipore Sigma, Burlington, MA) and p90 ribosomal S6 kinase (RSK) (1:1,000; #79-554; ProSci Inc., Poway, CA) were obtained from the corresponding listed company.

Cell culture. SNU-C5 (IC_{50} against 5-FU = 5 μM) cell line was purchased from the Korean Cell Line Bank (Seoul, Republic of Korea), and 5-FU-resistant SNU-C5 (SNU-C5/5-FUR; IC_{50} against 5-FU = 140 μM) cell line was obtained from the Research Center for Resistant Cells (Chosun University, Gwangju, Republic of Korea). Cells were cultured in RPMI-1640 medium (Welgene, Gyeongsan, Republic of Korea) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin at 37 ºC in a 5 % CO_2 incubator as previously described (Moon *et al*., 2020; Kim *et al*., 2021).

Cell viability assay. The effect of drugs on cell viability was evaluated by MTT reduction to its formazan product. SNU-C5 (2×10^3 cells/well) and SNU-C5/5-FUR (5×10^3 cells/well) cells were seeded in triplicate wells of 96-well plates, and treated with YE (15 mg/ml), Ko143 (25 nM), and 5-FU (at various concentrations). The cells were incubated for 3 days, and 10 μl of the MTT reagent (5 mg/ml in PBS) was added to each well, followed by incubation of the cells for 3 hours, and then dissolved in DMSO for 15 min. The MTT reduction was measured spectrophotometrically at 595 nm using the absorbance at 620 nm as the background with a VERSAmax microplate reader (Molecular Devices Korea LLC). The absorbance values obtained from the wells of the vehicle-treated cells represented 100 % cell viability and were used for comparison with the treated cells. The effect of the drugs was calculated and compared to the untreated cells.

Western blotting. Cells were treated with or without YE, 5-FU, and Ko143, for 3 days, followed by western blotting as described previously (Moon *et al*., 2020; Kim *et al*., 2021). To obtain the intracellular proteins, cultured cells were harvested in M-PER mammalian protein extraction reagent (ThermoFisher Scientific, Waltham, MA) including 1 %

protease inhibitor cocktail set III, 0.5 % phosphatase inhibitor cocktail 2, and 0.5 % phosphatase inhibitor cocktail 3 (Millipore Sigma). The protein concentrations were assessed using BCA protein assay (ThermoFisher Scientific) according to the manufacturer's instructions.

Electrophoresis of the protein in cell lysates was performed with the TGX Stain-Free FastCast™ Acrylamide Starter Kit (Bio-Rad Laboratories, Inc., Seoul, South Korea) using a Tris/glycine buffer system (Bio-Rad Laboratories) and transferred onto PVDF membranes. The membranes were blocked at room temperature with 5 % skim milk for 1 h and then incubated with primary antibodies overnight at 4 °C. After washing, peroxidase anti-mouse or anti-rabbit IgG antibodies (#PI-2000 and #PI-1000; Vector Laboratories, Inc., Burlilngame, CA) were applied for 1 h at room temperature. Next, Western Lightning Chemiluminescence Reagent (PerkinElmer, Inc., Waltham, MA) was used to detect the proteins. Anti-GAPDH antibody was used as a loading control on the stripped membranes. The bands were captured using Azure™ c300 (Azure Biosystems, Inc.) and quantified using the AzureSpot analysis software (version 14.2; Azure Biosystems, Inc.).

Statistical analysis. All data were compiled from a minimum of three replicate experiments. Data are expressed as the mean values ± SD. P<0.05 was considered to indicate a statistically significant difference as determined using the Student's paired t-test or one-way ANOVA followed by a Bonferroni post-hoc test (MS Excel 2016).

RESULTS

Autophagy responses in SNU-C5 and SNU-C5/5-FUR cells after YE treatment. Expression of the autophagy proteins was assessed by Western blotting when treated with YE for 3 days (Fig. 1 and Table I). Compared with vehicle-treated condition, activation of LC3 (II/I) was significantly observed in SNU-C5 cells. Although each LC3-I and LC3-II were increased, the activation of LC3 was not seen in SNU-C5/5-FUR cells (Fig. 1A).

The related Atg proteins were further checked to compare the different responses. Atg7 was significantly increased in SNU-C5 cells, but not changed in SNU-C5/5-FUR cells. Atg12-5 complex was considerably increased in both CRC cells. Atg16L1 was increased with significance in SNU-C5/5-FUR cells or not in SNU-C5 cells. Other proteins were not changed considerably (Fig. 1B).

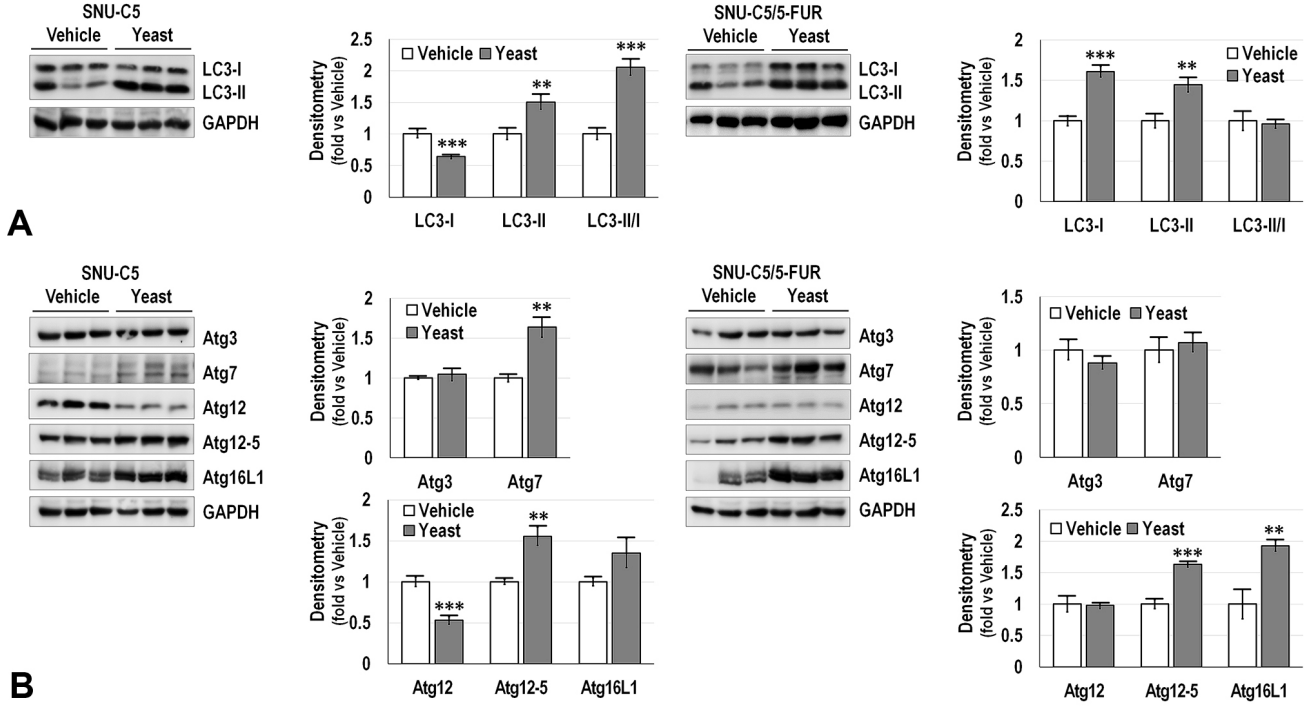

Fig. 1. Markers for autophagy in SNU-C5 and SNU-C5/5-FUR cells after yeast extract treatment. A. Expression levels of LC3, further divided into LC3-I and LC3-II, were detected by immunoblotting while GAPDH was used for a loading control. B. Expression levels of Atg proteins were detected by immunoblotting while GAPDH was used for a loading control.Band density was analyzed by AzureSpot analysis software, and results are expressed as the mean ± SD (n=3). **P<0.01 and ***P<0.001 vs. vehicle-treated condition

Necroptosis responses in SNU-C5 and SNU-C5/5-FUR cells after YE treatment. Expression of the necroptosis proteins was assessed (Fig. 2 and Table I).

The activation (phosphor/total) of RIP and RIP3 were not increased after YE treatment, but the activation of MLKL was slightly increased in both CRC cells with significance (Fig. 2A).

Caspase-8 was further divided into full (57kDa), intermediate (43kDa), and cleaved (18kDa) fractions. Each fraction was not considerably increased after YE treatment in both CRC cells. Activation of caspase-8 (cleaved/full) showed a significant increase in SNU-C5 cells, but not in SNU-C5/5-FUR cells (Fig. 2B).

Drug resistance-related markers in SNU-C5 and SNU-C5/5-FUR cells after YE treatment. Expression of the drug resistance-related markers was assessed (Fig. 3 and Table I). The expression of ABCG2 was significantly increased in SNU-C5 and SNU-C5/5-FUR cells after YE treatment. The expression of p90RSK was also considerably increased in both CRC cells.

Characteristics of SNU-C5/5-FUR cells on cell viability, proliferation, and drug-resistance. The characteristics of both CRC cells on drug resistance against 5-FU was assessed using the MTT assay. The difference in cell viability between both CRC cells was statistically significant: 92.2 ± 1.6 vs 97.9 ± 3.0 % at 1 mM ($p = 0.0442$) and 59.1 ± 1.6 vs 89.3 ± 2.6 % at 10 mM ($p < 0.001$). SNU-C5/5-FUR cells showed significantly slower proliferation than those in SNU-C5 cells (Fig. 4A and Table II).

SNU-C5/5-FUR cells showed an increased level of ABCG2 (1.78 ± 0.07 fold; $p < 0.001$) and a decreased level of p90RSK (0.49 ± 0.06 fold; $p = 0.0005$) when compared to SNU-C5 cells with significance, respectively. The activation of ERK (phosphor/total; 0.50 ± 0.02; $p = 0.0047$) was considerably decreased in SNU-C5/5-FUR cells (Fig. 4B).

Responses after Ko143, an ABCG2 inhibitor, treatment in SNU-C5/5-FUR cells. As compared with SNU-C5/5-FUR cells, co-treatment with 5-FU and Ko143 (25 nM) showed slightly lower survival rate. Cell viability was 70.4 ± 1.9 % vs 66.7 ± 0.8 % at 500 μM of 5-FU ($p = 0.0459$). With the condition, Ko143 treatment induced faster proliferation with or without 5-FU than those in SNU-C5/5-FUR cells (Fig. 5A and Table III).

When treated with Ko143 in SNU-C5/5-FUR cells, ABCG2 (0.59 ± 0.07 fold; $p = 0.0013$) was significantly suppressed without affecting p90RSK. The activation of ERK (0.63 ± 0.01 fold; $p = 0.0129$) was significantly decreased after Ko143 treatment in SNU-C5/5-FUR cells (Fig. 5B).

Table I. Densitometric results of western blotting on SNU-C5 and SNU-C5/5-FUR cells with yeast extract treatment.

Variables		SNU-C5	*p-value*	SNU-C5/5-FUR	*p-value*
A. Autophagy	LC3-I	0.64 ± 0.03	0.0002	1.61 ± 0.07	< 0.001
	LC3-II	1.50 ± 0.11	0.0032	1.44 ± 0.09	0.0027
	LC3-II/I	2.05 ± 0.13	< 0.001	0.96 ± 0.05	0.3882
	Atg3	1.05 ± 0.08	0.2997	0.88 ± 0.06	0.1457
	Atg7	1.64 ± 0.13	0.0011	1.07 ± 0.09	0.3196
	Atg12	0.53 ± 0.05	< 0.001	0.98 ± 0.05	0.4341
	Atg12-5	1.55 ± 0.11	0.0016	1.63 ± 0.05	< 0.001
	Atg16L1	1.35 ± 0.18	0.0522	1.93 ± 0.09	0.0050
B. Necroptosis	pRIP/RIP	1.05 ± 0.02	0.1589	1.03 ± 0.01	0.1475
	pRIP3/RIP3	1.03 ± 0.04	0.3003	0.90 ± 0.02	0.0024
	pMLKL/MLKL	1.10 ± 0.01	0.0055	1.23 ± 0.03	0.0002
	Caspase-8				
	Full	0.66 ± 0.03	0.0003	0.87 ± 0.08	0.0979
	Intermediate	0.75 ± 0.01	0.0028	0.86 ± 0.04	0.0106
	Cleaved	1.15 ± 0.09	0.1548	0.94 ± 0.06	0.1939
	Cleaved/Full	1.54 ± 0.11	0.0006	1.01 ± 0.13	0.4765
C. Resistance	ABCG2	1.63 ± 0.14	0.0006	1.19 ± 0.06	0.0143
	P90RSK	1.72 ± 0.06	< 0.001	2.65 ± 0.11	< 0.001

Each result was first normalized by GAPDH and then compared with the results obtained from vehicle-treated condition as a standard. Results were showed in terms of the relative fold with p value.

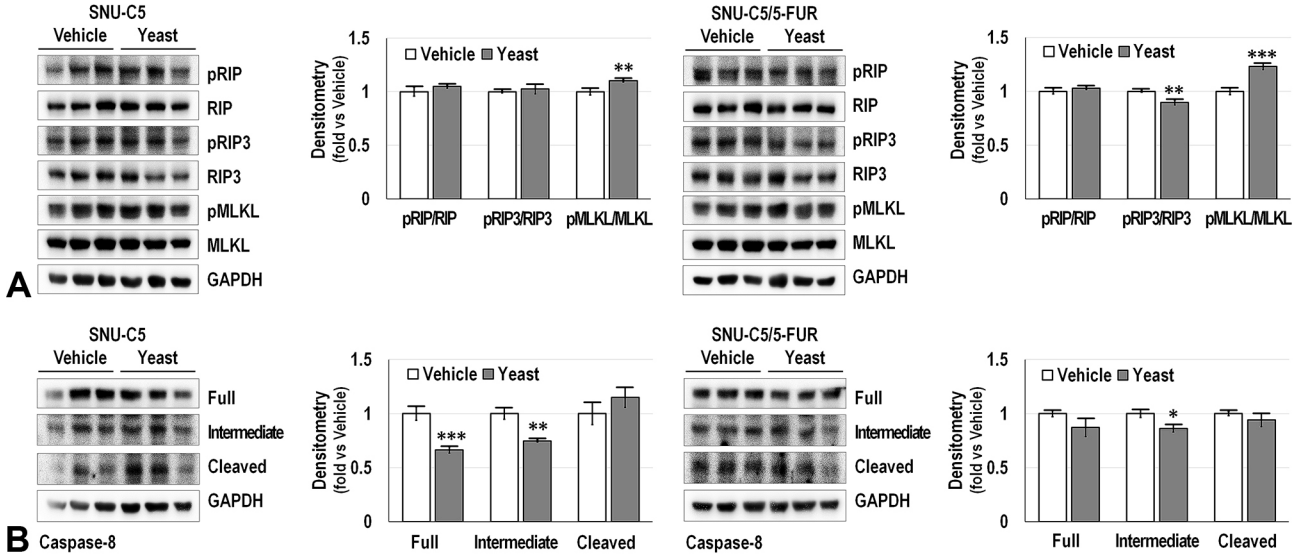

Fig. 2. Markers for necroptosis in SNU-C5 and SNU-C5/5-FUR cells after yeast extract treatment. **A.** Expression levels of RIP, RIP3, and MLKL were detected by immunoblotting while GAPDH was used for a loading control. **B.** Expression levels of caspase-8, further divided into full, intermediated, and cleaved fractions, were detected by immunoblotting while GAPDH was used for a loading control. Band density was analyzed by AzureSpot analysis software, and results are expressed as the mean ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle-treated condition

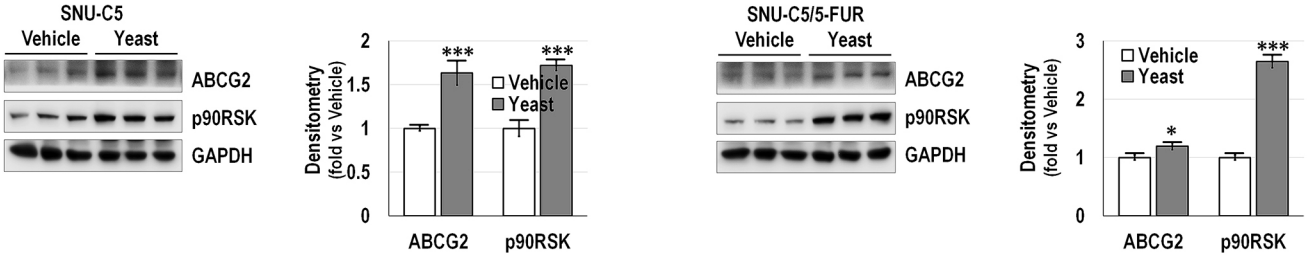

Fig. 3. Markers for drug-resistance in SNU-C5 and SNU-C5/5-FUR cells after yeast extract treatment. Expression levels of ABCG2 and p90RSK were detected by immunoblotting while GAPDH was used for a loading control. Band density was analyzed by AzureSpot analysis software, and results are expressed as the mean ± SD (n=3). *P<0.05 and ***P<0.001 vs. vehicle-treated condition

Table II. Relative proliferation of SNU-C5 and SNU-C5/5-FUR cells.

Variables	SNU-C5	SNU-C5/5-FUR	*p-value*
Day 1	1.00 ± 0.03	1.00 ± 0.05	
Day 2	2.08 ± 0.10	1.76 ± 0.07	*0.0085*
Day 3	3.79 ± 0.19	2.37 ± 0.10	*< 0.001*

The relative proliferation rates were obtained from vehicle-treated condition of Day 1 as a standard, and then compared between two cell lines.

DISCUSSION

We reported that YE has little effects on normal cells, but toxic effects on cancer cells (Moon *et al*., 2019, 2020). Cell cycle arrest and/or apoptosis would be the promising mechanisms for the anti-cancer effects. As there was little

Table III. Relative proliferation of SNU-C5/5-FUR cells with 5-FU and/or Ko143 treatment.

Variables	5-FU (0 μM)			5-FU (500 μM)		
	Ko143 (-)	Ko143 (+)	p-value	Ko143 (-)	Ko143 (+)	p-value
Day 1	1.35 ± 0.02	1.51 ± 0.03	0.0002	1.22 ± 0.02	1.57 ± 0.04	< 0.001
Day 2	1.85 ± 0.02	2.02 ± 0.04	0.0011	1.35 ± 0.03	1.62 ± 0.04	< 0.001
Day 3	2.76 ± 0.05	3.40 ± 0.10	< 0.001	1.44 ± 0.08	1.75 ± 0.06	0.0027

The relative proliferation rates were obtained from vehicle-treated condition of Day 0 as a standard (neither 5-FU nor Ko143 (25 nM)), and then compared between Ko143 treatments.

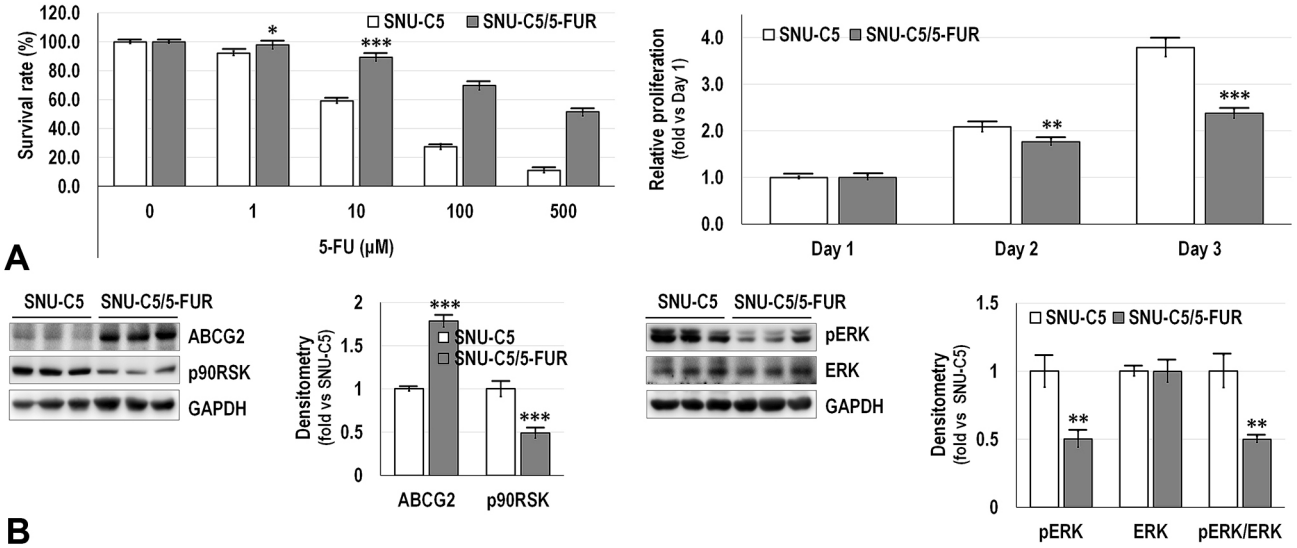

Fig. 4. Characteristics of SNU-C5/5-FUR cells as compared with SNU-C5 cells. **A.** The cells were mock-treated with DMSO or treated with indicated concentrations of 5-FU for 3 days (left) and cultured for indicated days (right). The extent of cell viability was determined by MTT assay. Data are presented as mean ± SD. *p < 0.05 and ***p < 0.001 vs. SNU-C5. **B.** Expression levels of ABCG2 and p90RSK (left), and the activation of ERK (right) was detected by immunoblotting while GAPDH was used for a loading control. Band density was analyzed by AzureSpot analysis software, and results are expressed as the mean ± SD (n=3). **P<0.01 and ***P<0.001 vs. SNU-C5

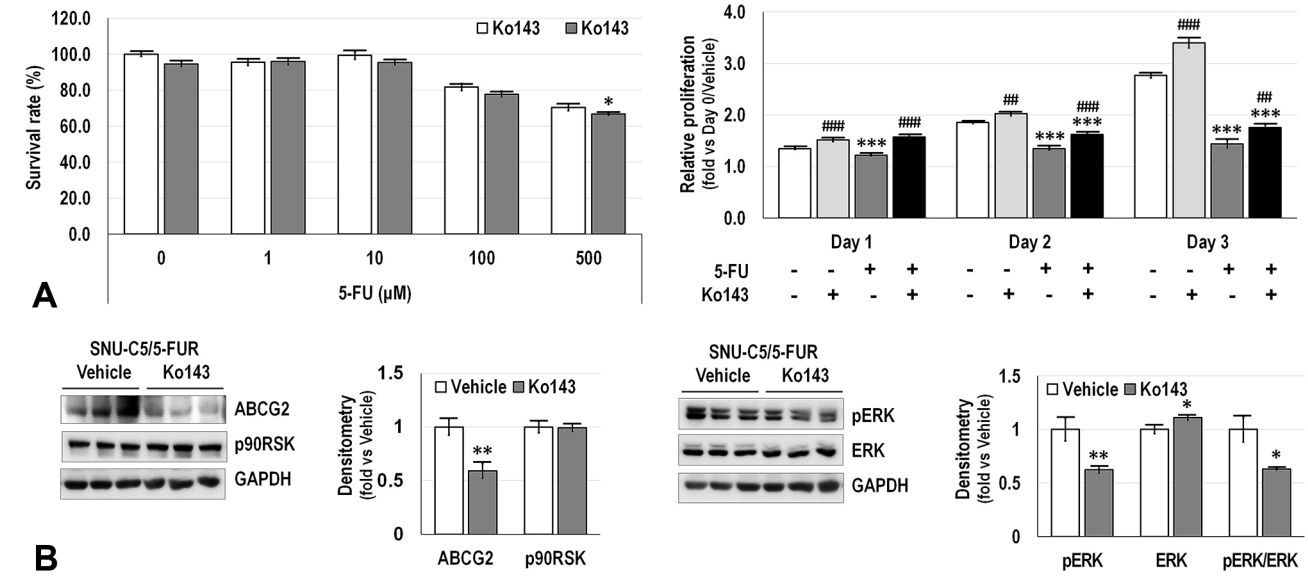

Fig. 5. Response after ABCG2 inhibitor (Ko143) treatment in SNU-C5/5-FUR cells. **A.** The cells were mock-treated with DMSO or treated with Ko143 (25nM) for 3 days (left) and cultured for indicated days with indicated concentrations of 5-FU with or without Ko143 (right). The extent of cell viability was determined by MTT assay. Data are presented as mean ± SD. *p<0.05 between Ko143 treatment (left), ***p<0.001 between 5-FU treatment (right); ##P<0.01 and ###P<0.001 between Ko143 treatment (right). **B.** Expression levels of ABCG2 and p90RSK, and the activation of ERK were detected by immunoblotting while GAPDH was used for a loading control. Band density was analyzed by AzureSpot analysis software, and results are expressed as the mean ± SD (n=3). *P<0.05, **P<0.01, and ***P<0.001 vs. vehicle-treated condition

difference on cell death responses under flow cytometry analysis between the wild type and 5-FU-resistant CRC cells under YE treatment, autophagy and necroptosis were further examined. SNU-C5 cells showed more susceptibility to LC3-activated autophagy through the increased Atg7 and Atg12-5 complex under YE treatment. Other cell death pathways including necrosis (Moon *et al*., 2020) and necroptosis in this study were not changed, and thus activated caspase-8

may induce apoptosis in SNU-C5 cells as previously suggested (Moon *et al*., 2020). As compared to SNU-C5 cells, SNU-C5/5-FUR cells did not show active autophagy despite the increased Atg12-5 complex and Atg16L1, and also did not induce necroptosis and caspase-8-dependent apoptosis. As a result, SNU-C5/5-FUR cells showed a relative resistance to autophagy under YE treatment.

Autophagy can play tumor-suppressive or tumor-promoting roles and facilitate malignant progression with increased drug resistance, which is determined by oxygen and nutrient availability and microenvironment stress (Li *et al*., 2020; Lim *et al*., 2021). In CRC, the expression of LC3 is significantly higher than the control and correlated with tumor aggressiveness, indicating a tumor promoting role of autophagy (Zheng *et al*., 2012). Although not CRC, inhibition of autophagy in cancer stem cells increased drug sensitivity to paclitaxel and cisplatin (Lim *et al*., 2021). Although a previous report (Yao *et al*., 2017) that 5-FU-resistant CRC cells showed reduced autophagy as compared to wild type SNU-C5 cells, SNU-C5/5-FUR cells might have potential of protective autophagy with increased LC3-II as well as LC3-I. The interpretation could be reinforced by a previous review (Azwar *et al*., 2021) that increased autophagy is suggested as one of the mechanisms of resistance to 5-FU in CRC.

Another mechanism of resistance to 5-FU, increased drug efflux was examined. ABCG2, also known as breast cancer resistance protein, is an ABC transporter that mediates energy-dependent transport of substrate drugs out of the cell and therefore its overexpression may contribute to intrinsic drug resistance (Zhang *et al*., 2022). The expression of ABCG2 was significantly increased in both CRC cells after YE treatment as 5-FU can induce the expression of ABC transporters in CRC (Gao *et al*., 2020). As the same responses on ABCG2 were observed in both CRC cells under YE treatment, the basic characteristics were evaluated. As compared with wild type SNU-C5 cells, SNU-C5/5-FUR cells showed relatively slower proliferation with increased ABCG2 expression and decreased phosphorylation of ERK as previously reported (Moon *et al*., 2020). ABCG2 inhibitor, Ko143, induced a relatively faster proliferation in SNU-C5/5-FUR cells, but did not significantly change on the viability against 5-FU with decreased phosphorylation of ERK.

According to the mitogen-activated protein kinase (MAPK) pathway, RSK is one of the downstream effectors of ERK (Katayama *et al*., 2016). ABCG2 expression is transcriptionally upregulated through the inhibition of the MAPK/ERK Kinase (MEK)-ERK-RSK pathway, and post transcriptionally downregulated through the inhibition of the MEK-ERK-non-RSK pathway (Imai *et al*., 2009). That is, ERK inhibition induces transcriptional upregulation and prompted protein degradation of endogenous ABCG2, and thus endogenous ABCG2 was eventually found to be upregulated, while p90RSK inhibition resulted in transcriptional upregulation of endogenous ABCG2 but did not affect the protein degradation of exogenous ABCG2. Similar to the previous report (Imai *et al*., 2009), SNU-C5/5-FUR cells showed upregulated ABCG2 and downregulated p90RSK and ERK phosphorylation.

Because ERK and RSK was downregulated in SNU-C5/5-FUR cells, ABCG2 expression was inhibited to reveal the linkage. Although verapamil, an inhibitor of ABCG2, inhibits proliferation in HT29 CRC cells (Nagheh *et al*., 2017), Ko143 enhanced proliferation in SNU-C5/5-FUR cells. Although the downregulation of ABCG2 induces a reversal of ABCG2-mediated drug resistance (Zhang *et al*., 2010; Mazard *et al*., 2013), the cell viability on 5-FU showed little changes. Ko143 treatment inhibited the expression of ABCG2 and ERK phosphorylation, but did not affect the expression of RSK. Although inhibition of ABC transporters is an important approach to overcome drug resistance (Gao *et al*., 2020), Ko143 on SNU-C5/5-FUR cells did not induce the expected effects. It is also known that inhibition of ABCG2 improved the efficacy of anti-cancer drugs by inhibiting ERK activation in CRC (Mazard *et al*., 2013), which was also observed in this study. Martinez-Lopez *et al*. (2013) propose that Atg12-5-positive preautophagosome and LC3-II-positive membranes facilitate MEK-ERK cascade and ERK phosphorylation. Similar results with increased Atg12-5 complex and ERK phosphorylation were observed in SNU-C5/5-FUR cells after YE treatment.

Taken together, protective autophagy in SNU-C5/5-FUR cells with increased ABCG2 expression might be candidate mechanisms for drug resistance. As the ERK responses were different from each stimulus, the feasible mechanisms among ERK-RSK-ABCG2 should be further investigated in 5-FU-resistant CRC cells.

YOON, S. P. Autofagia protectora en células de cáncer colorrectal resistentes al 5-fluorouracilo y enlace ERK-RSK-ABCG2. *Int . J. Morphol., 41(6)*:1816-1823, 2023.

RESUMEN: Para evaluar los efectos anticancerígenos del extracto de levadura en células resistentes, se investigaron la autofagia y la necroptosis en células de cáncer colorrectal resistentes al 5-fluorouracilo (5-FU). Además se evaluaron otras características subyacentes de la resistencia a los medicamentos centrándose en el enlace ERK-RSK-ABCG2. Se usaron células de cáncer colorrectal SNU-C5 (SNU-C5/5-FUR) resistentes a SNU-C5 y 5-FU para el ensayo de viabilidad celular y la transferencia Western para examinar los efectos anticancerígenos del extracto de levadura. El extracto de levadura indujo autofagia en células SNU-C5 con

mayor activación de Atg7, complejo Atg12-5, Atg16L1 y LC3 (LC3-II/LC3-I), pero pocos efectos en células SNU-C5/5-FUR con aumento de Atg12-5 complejo y Atg16L1. Ambas células de cáncer colorrectal no mostraron necroptosis después del tratamiento con extracto de levadura. Se evaluaron los mecanismos de resistencia a los medicamentos. en base al aumento de la expresión de ABCG2 y RSK después del tratamiento con extracto de levadura.En comparación con las de tipo salvaje, las células SNU-C5/5-FUR mostraron más expresión de ABCG2, menos expresión de RSK y menos fosforilación de ERK. El tratamiento con inhibidor de ABCG2, Ko143, induce los siguientes cambios: 1) más sensibilidad a 5-FU 500 mM, 2) proliferación aumentada y 3) menos fosforilación de ERK. Estos resultados sugieren que la autofagia protectora en células SNU-C5/5-FUR con mayor expresión de ABCG2 podría ser un mecanismo candidato para la resistencia a los medicamentos. Como las respuestas de ERK fueron diferentes de cada estímulo, los mecanismos factibles entre ERK-RSK-ABCG2 deberían investigarse más a fondo en células CCR resistentes a 5-FU.

PALABRAS CLAVE: Autofagia; Cáncer colorrectal; Resistencia a los medicamentos; ERK; 5-fluorouracilo.

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