이학박사 학위논문

Study on CEACAM1 Mediated Cell Death and Antitumor Effects of Metformin in 5-Fluorouracil Resistant Gastrointestinal Cancer Cells

5-Fluorouracil 내성 위장관암 세포에서 CEACAM1 매개 세포사멸 및 Metformin 항암효과에 관한 연구

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지도 교수 구 자 록

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김 성 희

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Study on CEACAM1 Mediated Cell Death and Antitumor Effects of Metformin in 5-Fluorouracil Resistant Gastrointestinal Cancer Cells

By

Sung-Hee, Kim

(Directed by Ja-Lok, Ku, D.V.M., Ph.D)

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Approved by Thesis Committee:

Professor _____ Chairman

Professor _____ Vice Chairman

Professor _____

Professor _____

Professor _____

ABSTRACT

Study on CEACAM1 Mediated Cell Death and Antitumor Effects of Metformin in 5-Fluorouracil Resistant Gastrointestinal Cancer Cells

Sung-Hee, Kim

Cancer Biology

The Graduate School

Seoul National University

Gastrointestinal (GI) cancer has high incidence and death rate in Korea. Thus to understand about tumorigenesis mechanisms and develop the new therapeutic strategies of GI cancer are important for reducing cancer risk. Conventional therapeutic strategies of GI cancers are surgery with chemotherapy, and chemo-radiotherapy. 5-fluorouracil (5-Fu), oxaliplatin, and irinotecan are most widely used for GI cancer.

The combination of 5-Fu with oxaliplatin or irinotecan has improved response rate about up to 40-50% for GI cancer patients. However, still the metastasis and recurrence that related with 5-Fu resistance are occurred, it might be needed of basic research about resistance mechanisms and new strategies for improve of therapeutic effects. In this paper, sensitivity to 5-Fu and gene expression pattern as treated with 5-Fu in parental cancer cell lines, SNU-638, SNU-C5, and their 5-Fu resistant cancer cell lines were investigated.

Based on this study, the purpose of paper is suggestion of adjuvant 5-Fu to recover the resistance. Because of Carcinoembryonic antigen-related cell adhesion molecule1 (CEACAM1), known as tumor suppressor gene, expression level was changed as treated with 5-Fu dose-, and time dependent manner in parental cell lines, I was supposed to that CEACAM1 might be correlated with 5-Fu sensitivity. However, oxaliplatin, irinotecan, and radiation affected to CEACAM1 expression level only in parental cancer cell lines. Through this, I suggested that increased CEACAM1 was one of the phenotypes of cell death regulated by chemotherapeutic agents. As a results, I suggested that CEACAM1 could be used as an indicator of chemotherapeutic agents mediated cell

death. Metformin, one of the type II diabetics therapeutic agent that was recently reported about anticancer effect. It was also induce CEACAM1 expression level in GI cancer cell lines. Beside of this, metformin has synergistic effect with 5-Fu especially in 5-Fu resistant cancer cell line. In addition, metformin inhibits cell proliferation, increase cell death and cell cycle arrest, and downregulation of cancer stem cell marker genes. These effects of metformin were concomitant of inhibition of DNA replication machinery and mitotic cell cycle genes. According to series of results, I proposed that use of metformin as adjuvant of 5-Fu that might be reduction of 5-Fu resistance, with less clinical severe adverse effects.

Key words: Gastrointestinal (GI) cancer, 5-Fluorouracil (5-Fu), oxaliplatin, irinotecan, 5-Fu resistance, Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), Metformin

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LIST OF TABLES AND FIGURES

PART II

TABLE OF CONTENTS

GENERAL BACKGROUND

Gastrointestinal cancer

Gastrointestinal (GI) cancers, including gastric cancer (GC), colorectal cancer (CRC), hepatocellular carcinoma (HCC), esophageal cancer (EC) and pancreatic cancer (PC) is high increasing incidence rate in Korea [1]. Especially, CRC is detected to the most common cancer in male for the first time in 2016, and third in women. It caused by westernized diet, alcohol drinking, and cigarette smoking [2]. Followed by CRC, stomach, lung, liver and thyroid cancer is high incidence rate in male, while thyroid, breast, stomach, and lung cancers are high ranked in women [1]. All of this taken together, to understand about gastrointestinal cancer is important for reducing cancer risk.

Gastric cancer Despite of there have been reduction tendency in gastric cancer (GC) incidence and mortality around the world in the last 50 years, however, it is still high in East Asia including Korea [1, 3]. GC remains asymptomatic in general, however, it is commonly early detection in Korea due to dissemination of heath examination [4].

Causes of GC are as followings; first, diet. A high consumption of salt, salted foods, pickled food, and dried fish are significantly associated with increased risk of GC [3, 5]. Second, *H. pylori* infection. The WHO classified *H. pylori.* as class I carcinogen of GC that based on epidemiological evidences in 1994. Since that, *H. pylori* is established as a major risk factor for GC enhanced by salt intake [5, 6]. Third, other environmental factors such as alcohol, tobacco, and general carcinogens are weak association with GC [3]. Genetic background is the forth cause of GC. Hereditary factors such as *cadherin 1* (CDH1) germline mutation, and *catenin α-1* (CTNNA1), causes under 3% of GC. Also acquired genetic factors influence to sporadic GC, which chromosomal instability, fusion genes *i.e. SLCIA2* (SLCIA2-CD44), microsatellite instability, and single-nucleotide polymorphisms (SNPs) [7].

Surgery accompanied with chemotherapies is common treat to GC [4]. Although surgery performed complete resection, there are still recurrence at 19-42% of cases [4]. Considering local recurrence, adjuvant chemotherapy has been arise. Mono-chemotherapy with fluoropyrimidines, such as 5-fluorouracil (5-Fu) was classical therapeutic strategy. Treatment with 5-Fu after surgery, 3-years

survival rate was increased to 80.1% compared to only performed surgery group (70.1%), and 5- years survival rate was 72.6% in limited result by patient selection [8]. To increase the effectiveness, XELOX, capccitabine plus oxaliplatin, FORFOX, 5-Fu/ leucoborin with oxaliplatin, FAMTX, 5-Fu, doxorubicin and methotrexate, that with or without radiotherapy were performed. These adjuvant chemotherapy improved prognosis and safe to patients [4].

In GC, more than 50% of patients have metastasis to lymph node at initially diagnosed or at surgical resection, which leads to 5-year survival rate under 50% [9, 10]. And early GC patients who had lymph node metastasis, had higher recurrence rate and short term survival rate. Recurrence is the most common reason for cancer related cell death in GC patients [9]. To improve the clinical effectiveness and prognostic in GC patients, needs to be further study of recurrence, metastatic markers and chemotherapeutic pathway.

Colorectal cancer Colorectal cancer (CRC) is upper ranked of diagnosis and death rate in worldwide. Especially in Korea, for the first time, CRC is the most common cancer in male, in 2016 [1]. CRC is traditionally divided into two groups, familial- and sporadic CRC and hereditary CRC.

About 10–30% of familial CRC is occurred by adenomatous polyposis coli (APC) germ line mutation followed K-ras mutation [11]. Individuals who carried a germline mutation of APC, the first hit, was eventually developed CRC by occurred of second hit, this called by loss of heterozygosity (LOH) [12]. Germline mutations in the mismatch repair (MMR) genes such as *MLH1, MSH2, MSH6* and *PMS2* cause hereditary non-polyposis colorectal cancer (HNPCC), with a penetrance of approximately 5–7% for CRC [12, 13]. Beside FAP and HNPCC, there are other hereditary CRC syndromes as shown in Text table 1.

Text table 1. Genes involved in hereditary colorectal cancer syndrome [11].

Syndrome	Gene	Hereditary
Hereditary non−polyposis colorectal cancer (HNPCC)	*MLH1, MSH2, MSH6, MLH3, MSH3 and PMS2*	Dominant
Turcot Syndrome (TS)	*MMR or APC*	Dominant or Recessive
Familial Adenomatous Polyposis (FAP)	*APC genes*	Dominant
MUTYH−associated polyposis (MAP)	*MUTYH*	Recessive
Peutz−Jeghers syndrome (PJS)	*STK11/ LKB1*	Dominant
PTEN hamartoma tumors syndrome (PHTS)	*PTEN*	Dominant
Juvenile polyposis syndrome (JPS)	*SMAD4− BMPR1A*	Dominant
Polymerase Proofreading−Associated Polyposis (PPAP)	*POLD1−POLE*	Dominant

About 70% of CRC is classified as sporadic CRC arise by somatic mutations [13]. Wnt/β-catenin pathway that acts as a cell-cell interaction and transcriptional activator, is the most frequently dysfunctional signaling in sporadic CRC patients. Due to Wnt signaling pathway advocate to cell proliferation and differentiation, it might be a significant effect on tumorigenesis [11]. In addition, PI3K/AKT, Ras/Raf, NF-κB, GSK-3β, and TGF-βRII signaling pathways are also involved in CRC [11-13].

At diagnosis of CRC, TNM classification of Malignant Tumors stages as followed; 15% of patients present with TNM stages 0 and I, 20–30% with stage II, 30–40% with stage III, and 20–25% with stage IV CRC [14]. In accordance with TNM staging, it might be needed proper therapeutic strategies to improve the survival rate. With surgical operation, adjuvant chemotherapies were used for CRC. First, a standard care is 5-Fu based therapy, since in the early 1990s [14]. To improvement of prognosis in patients, leucovorin (LV) was used with 5-Fu. Second, 5-Fu regimen with oxaliplatin and irinotecan. After treated 5-Fu with irinotecan, called as FORFIRI, 3-years survival rate was 65.1% and 61.8% without irinotecan at passed III trials [14, 15]. Combination

treatment with 5-Fu and irinotecan has less significantly therapeutic improvement, however, 5-Fu with oxaliplatin has more successful data. 5-Fu regimen with oxaliplatin, FOLFOX, has improvement in 3-year survival rate up to 78.2% compared with 72.9% that treated with 5-Fu/LV [16]. This results suggested that FORFOX might be used for new standard adjuvant chemotherapy. Third, there are expanded therapeutic strategies such as targeted epidermal growth factor receptor (EGFR) or vascular endothelial growth factor (VEGF), *e.g.* cetuximab, and bevacizumab, respectively that inhibited tumor growth and angiogenesis [14]. Forth, especially in rectal cancer, radiation therapy was performed. 5-Fu treatment with radiotherapy, improve the therapeutic effect in high-risk rectal cancer patients [17].

Approximately 50% of CRC patients with stage III has recurrence following curative surgery, tumor specific death related with metastasis with 5-year survival rates of 59.5% [1, 14, 18]. The liver was the major target organ of CRC metastasis with 78% rate, lung metastasis was found in 20% of patients, and bone and brain were under 5% [19].

Prevention of metastasis of CRC and improvement therapeutic efficiency based on new molecular targets are might be future studies.

Molecular pathway of therapeutic agents for GI cancer

Therapeutic strategies of gastrointestinal (GI) cancers are surgical resect of a lesion, pre-, and post-operative chemotherapy, radiotherapy, and chemo-radiotherapy. Performed surgery with chemotherapy, either chemo-radiation are improved outcome and prognosis [4, 14]. There are attempts to improve effect of conventional therapeutic agents, 5-fluorouracil (5-Fu), via drug combinations. Oxaliplatin, and irinotecan are typical drugs that used combined with 5-Fu. Many clinical trials were performed using these drug combination, and has positive effect of curation [4, 15-17, 20, 21]. Despite of the curative effectiveness, recurrence and metastasis are still at a high risk.

5-Fu, oxaliplatin, irinotecan, and radiation has several working pathway, and to understand the mechanisms is important for combination therapy.

5-Fluoriuracil (5-Fu) 5-Fu is an analogue of uracil (U) that has substitution hydrogen to fluorine at C-5 position, whenever it enters to the cells it worked with the same transport system of uracil [22]. In cells, 5-Fu is converted to 3 main metabolites that fluorodeoxyuridine

monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) [22].

5-Fu metabolites disrupt RNA synthesis. FdUTP misincorporation into normal RNA process and disrupts its function. It can be affected to inhibition of pre-rRNA into mature form, splicing of mRNA, and post-transcriptional modification of tRNA. As a result, cell damage is induced by accumulation of RNA damages [22].

Also, 5-Fu induces inhibition of Thymidine Synthase (TS). FdUTP alternatively binds to the nucleotide binding site of TS instead dUMP, which induced inhibition of dTMP synthesis. Consequentially, it causes imbalance of dNTP pool and increased level of dUMP. As a results, DNA replication and repair are inhibited and DNA damage is arise [22].

5-Fu is widely used for treatment of various cancers, including GI cancers, nonetheless, the overall response rate of 5-Fu based chemotherapy is only 10-15% [23]. This low therapeutic effectiveness caused by resistance, thus drug resistance becomes a critical concern for chemotherapy. Therefore, modulation of 5-Fu activation pathway is important for anticancer effect and clinical outcome.

CH_2THF is necessary for binding of FdUMP to TS, the concentration of CH_2THF on cells is increased as treated with leucovorin (LV, 5′ – formyltetrahydrofolate). Therefore, adjuvant chemotherapy of 5–Fu/LV is increased therapeutic effect up to 23% compared to single treatment of 5–Fu [22]. With these reasons, 5–Fu/LV is generally used chemotherapy strategy. The other combination of 5–Fu with oxaliplatin and irinotecan has been suggested that improved response rate about 40–50% for advanced CRC patients [16]. However, still the metastasis and recurrence that related with 5–Fu resistance are occurred, it might be needed of new strategies for improve of therapeutic effects and basic research about resistance mechanisms.

Oxaliplatin Oxaliplatin (cis–[(1R,2R)–1,2–cyclohexanediamine–N,N′] oxalato (2–)–O,O′] platinum) is a kind of platinum based drug used for treatment of metastatic CRC in combination with 5–Fu/LV [24]. Oxaliplatin has DNA damaging mechanisms of action; via formed DNA lesion. Oxaliplatin can induces 3 types of DNA crosslink lesion that intra–, inter– strand crosslink and DNA–protein crosslink [25]. These are inhibits DNA synthesis, repair and cell cytotoxicity. Compare to

conventional platinum drugs, such as cisplatin, oxaliplatin showed retained activity against cancer cells, thus anticancer effects are sustained for a long time than cisplatin [21].

Early clinical trials with single agent treatment of oxaliplatin have showed that only 10% of patients were responded. But, the response rate of combination treatment with 5-Fu/LV with oxaliplatin is dramatically improved about 58% [21]; these combination therapy is called as FOLFOX. Clinical trials of FOLFOX indicated that response rate, progression, survival rate, and metastasis were improved, with this regards, oxaliplatin became to be approved by the food and drug administration (US FDA) in 2002 [14, 15, 18, 21].

Irinotecan In human, irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; CPT-11) is hydrolyzed by carboxylesterases (CES) type 1 and 2, and transformed to its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin) [26]. Activated irinotecan is bind, and inactivated of DNA topoisomerase I complex, thus prevention of resealing of single strand breaks, like replication forks.

This ultimately acts as an anti-tumor agents on rapidly dividing cells and tissues [26]. FOLFIRI, combinational therapeutic strategies with 5-Fu/LV, and irinotecan has been used for improved clinical implication. FOLFIRI has a significant survival advantages as well of FOLFOX [14, 20].

OBJECTIVES

In this paper, I investigated sensitivity to chemotherapeutic agent, 5-Fu, in parental cancer cell lines and their 5-Fu resistant cancer cell lines. Then, to understand differences between these cell lines, I examined gene expression pattern in naïve parental-, 5-Fu resistant cell lines and as treated with 5-Fu, especially focused on CEACAM1 that known as a tumor suppressor gene. Finally, I intended to suggest the possible way to increase sensitivity to 5-Fu, in 5-Fu resistant cancer cell lines.

PART Ⅰ. Study of expression level of CEACAM1 and identification as indicator of cell damage

Abstract

Gastrointestinal cancer (GC) has high incidence and death rate in Korea. Chemotherapeutic strategies and radiation therapy were classical therapeutic approaches for GC, however, that strategies has limitations caused by resistance mechanisms. 5-fluorouracil (5-Fu) is widely used to treatment of GC, and combination treatment with oxaliplatin and irinotecan were used to improve of therapeutic effectiveness. In this study, I focused carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), one of the affected genes from chemo-resistance. CEACAM1 was highly expressed most of GC cell lines. In SNU-638, -C5 parental-, and 5-Fu resistant cell lines had differently CEACAM1 expression pattern, which higher in parental cell lines and lower in 5-Fu resistant cancer cell lines. As treated with 5-Fu, CEACAM1 was increased by dose-, and time-dependent manner especially in parental cell lines. Thus I speculated that CEACAM1 was correlated with 5-Fu sensitivity. Moreover, oxaliplatin, irinotecan, and radiation affected to CEACAM1 expression level only in parental cancer

cell lines not in 5-Fu resistant cancer cell lines. While, metformin dose not correlated with CEACAM1 expression level. Moreover, expression level of CEACAM1 was not correlated with cell proliferation rate, cell migration, and cell cycle *per se*. Through this, I suggested that increased CEACAM1 was one of the phenotypes of cell death regulated by chemotherapeutic agents. In this study, I suggested that CEACAM1 could be used as an indicator of effect for chemotherapeutic agents, especially 5-Fu.

Keywords: Gastrointestinal cancer (GC) cell lines, drug resistant cell lines, 5-fluorouracil (5-Fu), oxaliplatin, irinotecan, radiation, CEACAM1

Introduction

Gastrointestinal cancer has high incidence rate, at a third level in male, and a second level in female, and estimated cancer rate and death rate was the most higher than other cancers in Korea [1]. Chemotherapy and radio-therapy were classical anticancer therapeutic approaches for gastrointestinal cancer, however, that strategies has limitations for resistance mechanisms [27]. 5-fluorouracil (5-Fu) is widely used to treatment of gastrointestinal cancer. It is an analogue of Uracil (U); as transported to cells 5-Fu converted U to fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). These are active metabolites disrupt RNA synthesis that causes cell cytotoxicity and death [22]. In spite of the effectiveness of 5-Fu, the response rate of 5-Fu alone was only 10-15%, and combination with 5-Fu and other chemotherapeutic agents, oxaliplatin, irinotecan, was 40-50%. This limited effects were caused by resistance [28]. 5-Fu resistance arises from many reasons, 1)

alteration of drug in−, efflux through a variety of drug transporter that localized in cellular membrane, 2) modulation of metabolic pathway, intracellular catabolism, 3) epigenetically changes by DNA methylation, histone acetylation and other reasons, 4) presence of subpopulation i.e. cancer stem cells that possesses cancer initiation ability and self−renewal capacity [27, 29, 30]. Especially, one of the drug transporter, multidrug resistance (MDR) is a problematic in drug resistance. It is belongs to a family of ABC transporter, regulates drug influx and efflux, and the drug sensitivity in several cancers [31, 32]. Also, higher expression of MDR1 is one of the hall mark of cancer stem cells [32]. Since that, investigation of MDR and cancer stem cell might be need to increased sensitivity of anticancer agents.

As overexpressed MDR−1, cell proliferation and growth rate were changed. Even, it may induced stem cell rich tumor *in vivo* [33]. These caused by changed global gene expression, especially E−cadherin and carcinoembryonic antigen−related cell adhesion molecules (CEACAM) 1 [33]. Through this, these genes suggested that responsible for drug resistance. Among this, I focused on CEACAM1 that expression level was correlated to carcinogenesis of colorectal cancer [34].

CEACAM1, also known as BGP or CD66a, belong to CEA family and a subgroup of the immunoglobulin (Ig) superfamily. It has various isoforms that formed by transcriptional splicing variants, largely divided subgroup into a long (L) form that included phosphorylation sites containing of 71-73 amino acids, and short (S) form lacking of phosphorylation sites (10 amino acids) [35]. Due to expression of CEACAM1 was down regulated in several malignant cancers, it suggested that tumor suppressor gene, and upregulation was associated with a short survival rate and metastasis in some tumors [36-39]. Also, CEACAM1 acts as a regulator of apoptosis in the colonic epithelium which leads to caspase activation including caspase-1 and -3, and also involves non-caspase proteases; this was refer to CEACAM1-mediated cell death pathway [36, 38].

With this knowledge, I studied the correlation between CEACAM1 expression level and sensitivity to anticancer agent, 5-Fu, in Korean human gastrointestinal cancer cells. Through this, I aimed at define the mechanisms of CEACAM1-mediated-cell death, related to 5-Fu.

Materials and methods

Cell culture and Chemicals

Fourty-four human cancer cell lines and 7 pairs of parental-, 5-Fu resistant gastrointestinal cancer cell lines (Table 1, 2) that obtained from Korean Cell line Bank, Seoul, Korea.

All cell lines were maintained in RPMI1640 media except the CACO-2 cell line; maintained in Minimum Essential Medium, and the WiDr cell line; maintained in Dulbecco's modified Eagle's medium. All media were supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (FBS, Gibco, MA, USA). Cells were maintained in humidified incubators at 37° C, with an atmosphere of 5% CO_2 and 95% air. The 5-Fu resistant cell lines were established as described previously [40, 41], treated with 5-Fu (Sigma- Aldrich, MO, USA).

Oxaliplatin, irinotecan, and metformin were obtained from Sigma-Aldrich.

Table 1. List of colorectal cancer cell lines

Cell lines			
SNU-61	SNU-796	SNU-1544	DLD1
SNU-70	SNU-977	SNU-1684	HCT 8
SNU-81	SNU-1033	SNU-1746	HCT 15
SNU-175	SNU-1040	SNU-C1	HCT 116
SNU-254	SNU-1047	SNU-C2A	LoVo
SNU-283	SNU-1141	SNU-C4	Ls174T
SNU-407	SNU-1181	SNU-C5	NCI-H716
SNU-479	SNU-1197	Caco2 *	SW403
SNU-503	SNU-1235	Colo201	SW480
SNU-769A	SNU-1406	Colo205	SW1116
SNU-769B	SNU-1460	Colo320	WiDr **

* Maintained in MEM

** Maintained in DMEM

Other cell lines maintained in RPMI1640

Table 2. List of 5-Fu resistance cell lines

Parental Cell lines	5-Fu Resistant Cell lines
SNU-216	SNU-216_5FuR
SNU-601	SNU-601_5FuR
SNU-638	SNU-638_5FuR
SNU-769A	SNU-769A_5FuR
SNU-C2A	SNU-C2A_5FuR
SNU-C4	SNU-C4_5FuR
SNU-C5	SNU-C5_5FuR

Table 3. List of primer sets

Gene	Sequence
CEACAM1 F	3' AGCAACTGGACAGTTCCATGTATA 5'

CEACAM1 R	3’	AGGTAGGTTGTGTCCTGAGTCT 5’
CEACAM1_iso F	3’	GGTTGCTCTGATAGCAGTAG 5’
CEACAM1_iso R	3’	AGCCTGGAGATGCCTATTAG 5’
β-actin F	3’	GAGACCTTCAACACCCCAGC 5’
β-actin R	3’	GCTCATTGCCAATGGTGATG 5’

PCR and Western blot analysis

Total RNA isolation was used by easy-BLUE™ kits (Intron biotechnology, Gyeonggi, Korea). 2 μg of total RNA were combined with a random primer, dNTP, and 200 units of Superscript™ Ⅱ reverse transcriptase (Invitrogen, CA, USA) in a final volume of 20 μl for cDNA synthesis. The mixture incubated for 60 min at 42° C, after that incubated for 15 min at 70° C for denaturation. Next, 80 μl of distilled water was added to the reverse-transcription reaction mixture. PCR was performed to obtain mRNA expression data. The prepared 15 μl PCR mixtures included 1 μl of cDNA, 10× buffer, 2.5 mM of dNTP, 0.1 pM of primers and 1 unit of Taq DNA polymerase (Intron biotechnology). PCR amplification was conducted using the described primer sets (Table 3). The β-actin was used as an endogenous reference for the

normalization of expression levels. The following PCR conditions were used: initial denaturation for 5 min at 94° C, cycling at 94° C (30 sec), 55° C (30 sec), and 72° C (30 sec) for 35 cycles, with final elongation for 7 min at 72° C. PCR was performed in a thermal cycler (PCR System 9700, Applied Biosystems; CA, USA). The PCR products were divided in 2% agarose gel stained with ethidium bromide.

Cells were harvested using cell scrapper for protein analysis. Harvested cells were washed in PBS for three times, suspended in PRO-PREP™ kits (Intron biotechnology) and placed on ice for 30 min. After centrifuging at 13000 ×g for 30 min at 4° C, the supernatant was collected. Protein quantitative analysis was accomplished by using SMART™ micro BCA kit (Intron biotechnology). 20 μg of protein and 4× SDS sample buffer (Invitrogen) mixtures were boiled at 95° C for 5 min and loaded on 4-12% Bis-Tris gel (Invitrogen) at 100 V for 3 h. Proteins were transferred to a PVDF membrane (Invitrogen) by electro-blotting at a constant 270 mA current for 2 h. For membrane blocking, the membrane was incubated for 1 h at room temperature in 1.5% non-fat dry milk and 0.5% Tween 20-TBS buffer containing 1mM of $MgCl_2$. Primary antibodies against CEACAM1 (Abcam, Cambridge, UK)

(1:2000), caspase-3 (Abcam) (1:1000), PARP (BD, NJ, USA) (1:1000), E-cadherin, N-cadherin, FANCD2, and gamma-H2A.X (Abcam) (1:1000), mTOR, phosphor-mTOR, AMPKα, phosphor-AMPKα (BD) (1:1000), actin and tubulin (Abcam) (1:5000) were incubated overnight at 4° C. For secondary antibody incubation, peroxidase conjugated mouse or rabbit IgG antibody (Jackson Immunoreasearch, MD, USA) was diluted 1:5000. After incubation at room temperature for 1 h, a chemiluminescent working solution, WEST-ZOL™, (Intron biotechnology) was decanted to the membrane. The membrane was then covered with a thin plastic wrap and exposed to Fuji RX film for 1-60 min.

Cell proliferation assay

Cell proliferation was analyzed by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (Sigma- Aldrich) assay.

Initially, 2×10^5 cells were seeded on 96-well plates and cultured for overnight. Subsequently, media containing with 5-Fu were added to the plates and the cells incubated for 72 h before being treated with 50 μl of MTT solution for 4 h at 37° C. Absorbance at 540 nm was measured by

an MULTISKAN FC Microplate Photometer (Thermo-Fisher Scientific, MA, USA).

Establishment of shCEACAM1 cell lines

For establishment of CEACAM1 knockdown cell lines, I used short-hairpin (sh) RNA obtained from Sigma-Aldrich. The target sequence was 5'-CCATCATGCTGAACGTAAACCTCGAGGTTTACGTTCAGCATGATGG-3', which suppressed all variants of CEACAM1. Lentivirus production using 293T cells according to manufacturer's protocol; transfection with recommended ratio, lipofectamine 2000™ (Invitrogen) with shRNA: pCMV-VSV-G: pRSV-REV: pCgpV at 3:1:1:1 to 80% confluence cells. Lentivirus component were obtained from Cell Biolabs (San Diego, CA, USA). Then harvest lentiviral supernatant at 24, 48, and 72 h after transfection. 0.22 μM filtered supernatant was treated to SNU-638, SNU-C5 cells with ViraDuctin™ Lentivirus Transduction Kit (Cell Biolabs) to improvement of efficiency. After transduction, cells were selected with puromycin (Sigma-Aldrich).

Cell cycle analysis

For cell cycle distribution analysis, cells were fixed overnight in 70% ethanol at −20° C. Subsequently, cellular DNA was stained with 100 μg/mL of propidium iodide (PI) (Sigma−Aldrich) for 30 min on ice, then add 1 μl RNase A, 10 min at RT. After staining, cells were subjected to flow cytometer (FACS CantoII™, BD, NJ, USA) analysis of DNA content to determine the percentage of the cells in different cell phases and in apoptosis.

Wound healing assay

For cell migration assay, 1×10^6 cells were seeded on 24−well plate that contained wound−healing insert (Cell biolabs), and incubated cells in incubator for overnight. The insert were carefully removed and incubated with cell culture medium containing 5−Fu. 400 × magnification of images were taken at the initial time of wound and after 6, 24, 48, 72, and 78 h using a CCD camera (Olympus, Tokyo, Japan).

Statistical analysis

For statistical analysis, GraphPad Prism 5.03 (GraphPad Software, CA,

USA) was used. The mean ± SD was determined for experiments.

Results

CEACAM1 expression level in gastrointestinal cancer cell lines

To confirm the basal expression level of CEACAM1, I investigated the expression in 44 colorectal cancer cell lines and 7 pairs of parental and 5-Fu resistant gastrointestinal cancer cell lines. CEACAM1 gene was highly expressed most of gastrointestinal cancer cell lines (Figure 1). I also examined CEACAM1 expression level in parental-, and 5-Fu resistant cell lines whether there was difference (Figure 2). SNU-216, -769A, -C2A and paired 5-Fu resistant cell line were merely expressed CEACAM1 gene and protein, on the contrary SNU-601, -C4 and their 5-Fu resistant cell line were highly expressed CEACAM1. Interestingly, in SNU-638 and -C5 parental cell lines and 5-Fu resistant cell lines were differently expressed CEACAM1: highly

expressed in parental cell lines and low or merely expressed in 5-Fu resistant cell lines. Due to the dissimilarity between parental and 5-Fu resistant cell lines, I used SNU-638, and -C5 sets for further experiments to study the correlation between CEACAM1 expression level and sensitivity to 5-Fu.

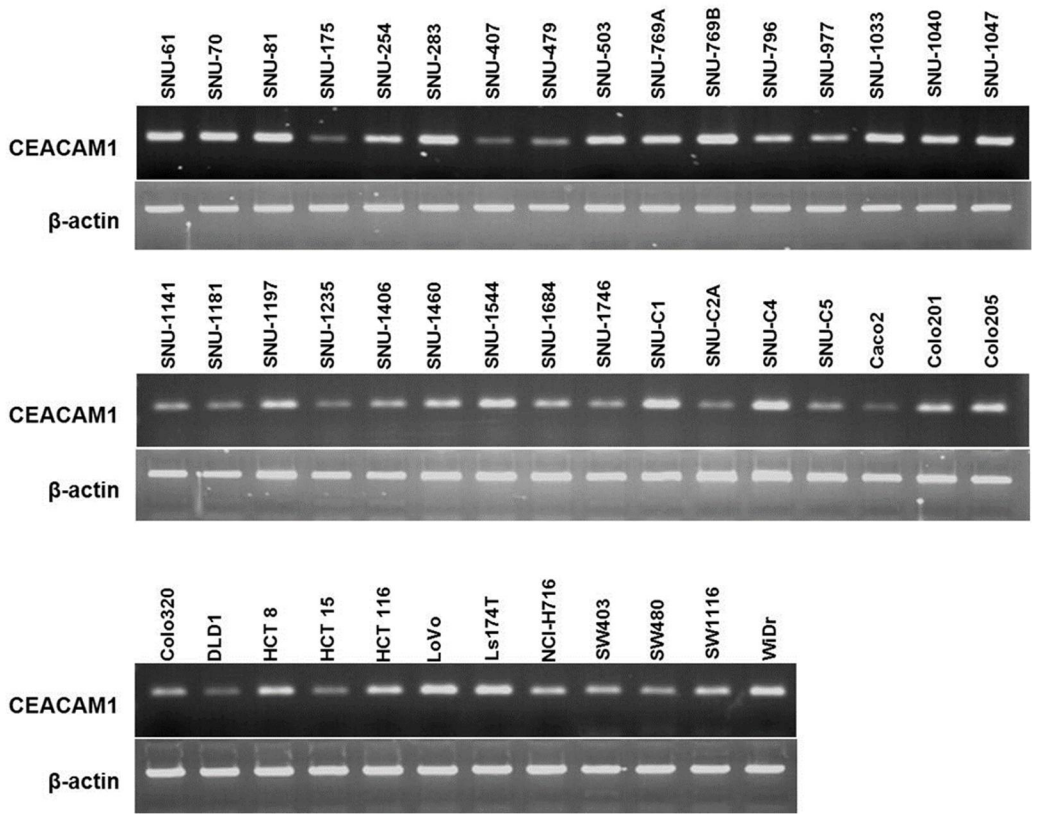


Figure 1. CEACAM1 expression level in colorectal cancer cell lines.

Gene expression levels are determined by RT-PCR, and beta-actin

used as internal control. CEACAM1 gene was expressed in most of 44 colorectal cancer cell lines.

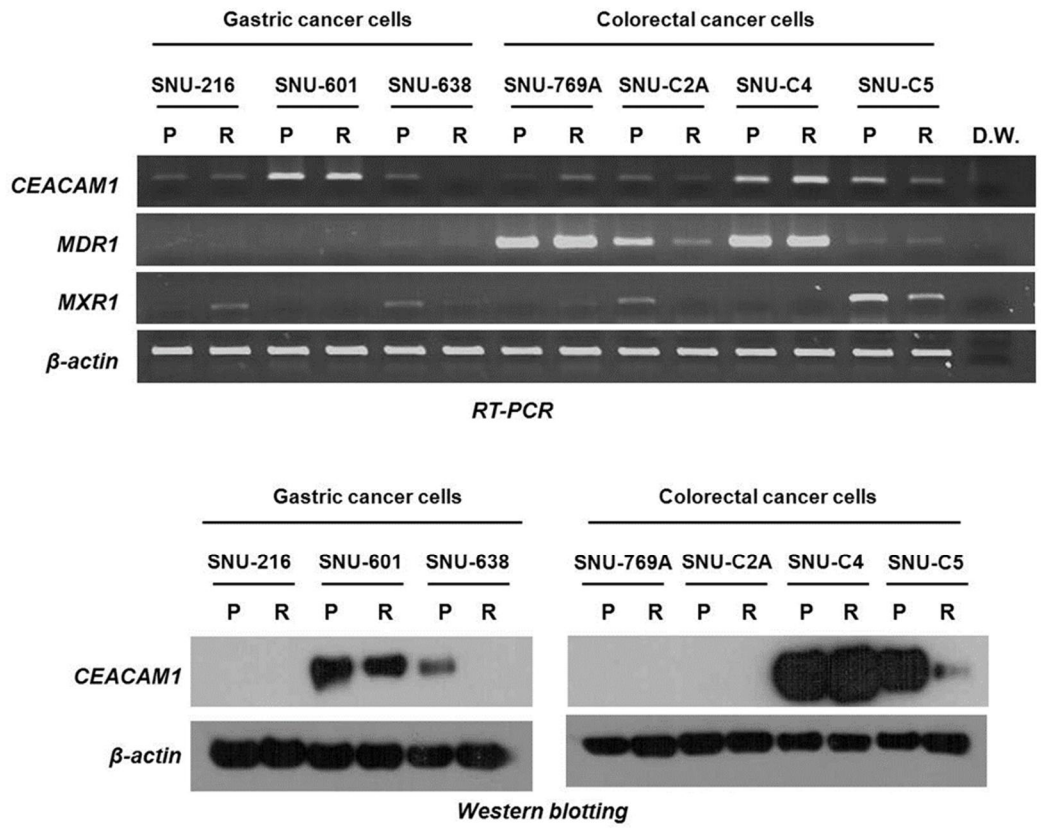


Figure 2. CEACAM1 expression level in gastrointestinal cancer cell lines, and their 5-Fu resistant cell lines.

In parental-, and 5-Fu resistant cell lines, CEACAM1 expression levels

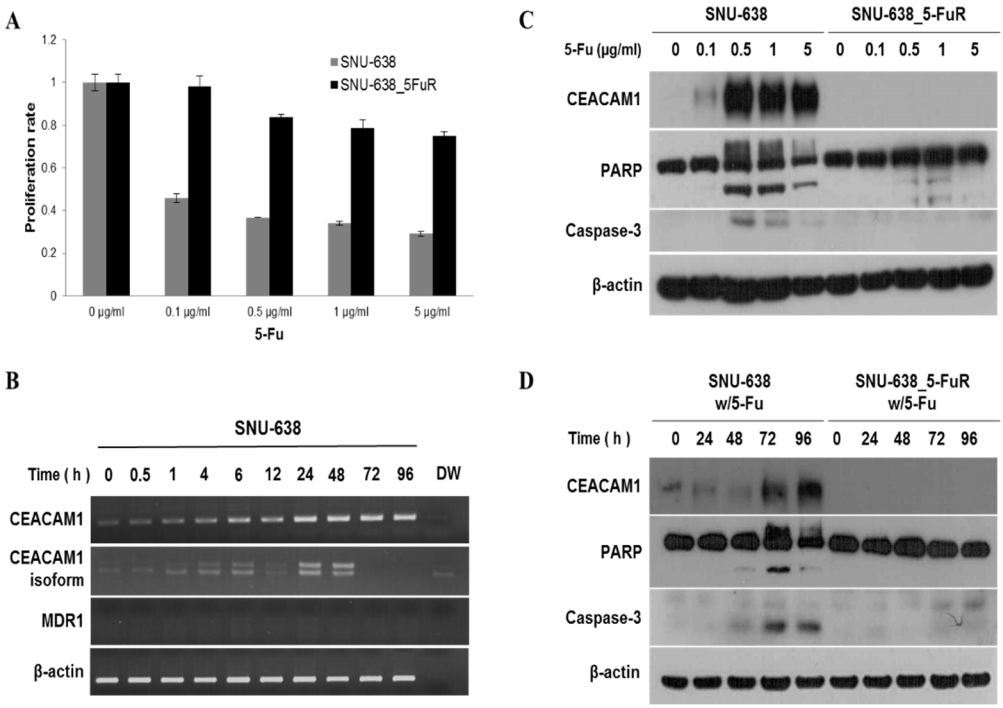
was determined by RT-PCR (A) and western blot analysis (B). Beta-actin used as internal control.

P: parental cell lines, R: 5-Fu resistant cell lines

CEACAM1 expression level was correlated with 5-Fu sensitivity

As treated with 1, 10, 50, and 100 μg/mL of 5-Fu, cell proliferation was significantly reduced in parental gastrointestinal cancer cell lines, SNU-638, and SNU-C5, whereas proliferation rate of 5-Fu resistant cell lines were not such significant; over 50 % reduction in parental cell lines and lesser than 25 % reduction in 5-Fu resistant cell lines as treated with 100 μg/mL of 5-Fu (Figure 3 A, E). Then CEACAM1 expression level of parental, 5-Fu resistant cells as treated with 5-Fu was confirmed. As shown in Figure 3, CEACAM1 expression level was low in 5-Fu resistant cell lines that merely affected to 5-Fu, however, CEACAM1 was increased as treated with 5-Fu dose-, time-dependent manner in SNU-638, SNU-C5 parental cell lines. To verified 5-Fu induced cell death, I confirmed apoptotic proteins, PARP cleavage and caspase-3. Similar with CEACAM1 expression pattern, PARP and caspase-3 increased as 5-Fu dose-, time-dependent manner,

especially in parental cell lines. These results suggested that CEACAM1 expression level was correlated with 5-Fu sensitivity. Above this, I suggested that treated 5-Fu induced CEACAM1-mediated cell death.



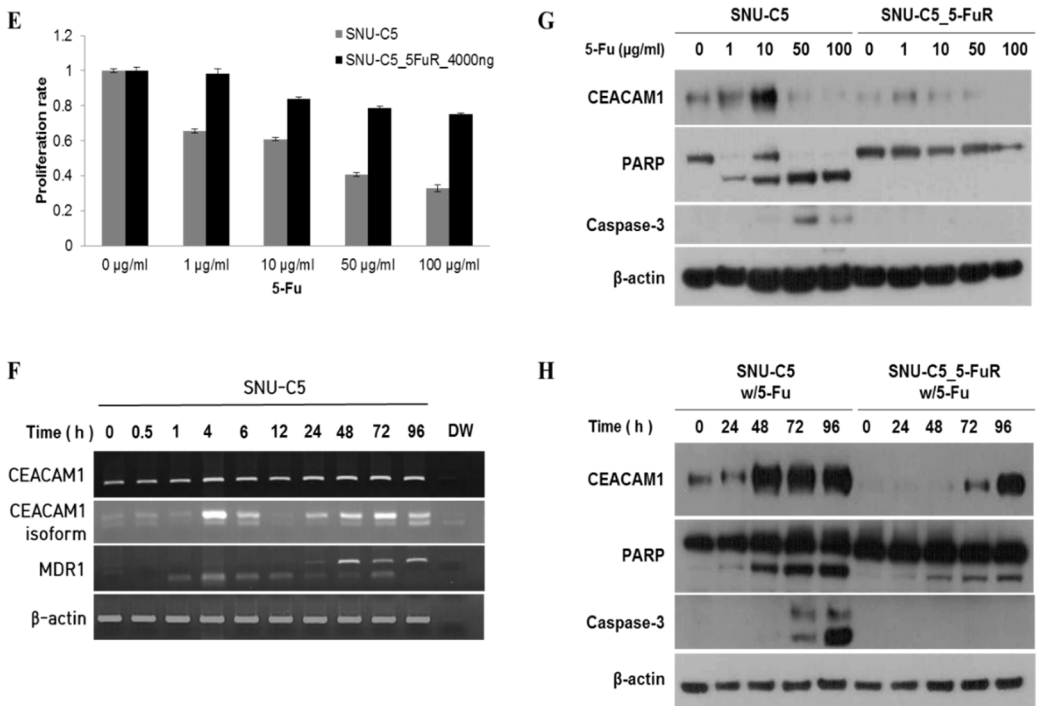


Figure 3. 5-Fu sensitivity and expression level of CEACAM1 in gastric (A-D), colorectal (E-H) cancer cell lines.

A and E represent 5-Fu cell proliferation assay in SNU-638 and SNU-C5 parental and resistant cell lines by cell proliferation assay. The data are represented as mean ± SD three independent experiments. Increased expression levels of CEACAM1 when treated with 5-Fu, are confirmed by RT-PCR (B, F) and western blot analysis by dose-(C, G), time-(D, H) dependent manner.

CEACAM1 was not directly correlated with 5-Fu mediated cell death

I constructed CEACAM1 knockdown cell lines to prove 5-Fu induced CEACAM1-mediated cell death. shCEACAM1 targeted whole isoforms of CEACAM1, this was effectively inhibited CEACAM1 expression in SNU-638 cell line, not in SNU-C5 (Figure 4). CEACAM1 downregulation was not correlated with cell proliferation rate, expression of cadherin and apoptotic molecules, cell cycle distribution, and wound healing ability (Figure 5-8, Table 4). I examined cell proliferation for several times, however, there was no repeatedly results (Figure 5). This means that CEACAM1 expression level was not a key factor of cell proliferation rate as treated with 5-Fu.

Singer, B. B. *et al*, suggested N-cadherin plays a role in the CEACAM1-mediated inhibition of cell adhesion [42]. Based on this research, I confirmed the cadherin to ascertain CEACAM1 that mediated in cell adhesion, and association with 5-Fu. In our study, expression of E-, N-cadherin were not changed as treated with 0, 100, 500 ng/mL of 5-Fu, and these genes were not correlated with CEACAM1 expression level

(Figure 6). Also, cell cycle distribution was not affected to CEACAM1 expression level and 5-Fu treatment, and SNU-638 parental cell line and shCEACAM1 had similar distribution of cell cycle that differ from 5-Fu resistant cell line (Figure 7). On the other hand, wound healing ability affected to CEACAM1 expression level; increased wound healing as inhibited CEACAM1 expression (Figure 8).

It was indicated that 5-Fu inhibits cell proliferation, however, does not induction of CEACAM1-mediated cell death, and CEACAM1 expression level was not correlated with cell death, *per se*. Thus I suggested that CEACAM1 was a phenomenon of cell death.

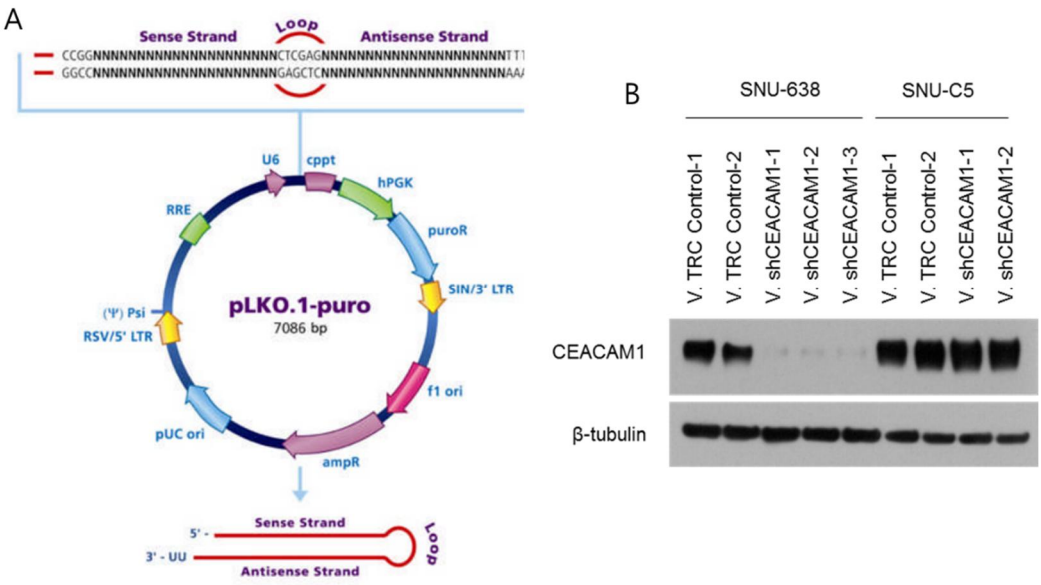


Figure 4. Establishment of CEACAM1 knockdown cell lines.

Constructed shCEACAM1 vector and control vector are purchased at Sigma-Aldrich. A represents shRNA vector map adopted from vendor (http://www.sigmaaldrich.com), and the target sequence of CEACAM1; 5' -CCATCATGCTGAACGTAAACCTCGAGGTTTACGTTCAGCATGATGG-3'. Established shCEACAM1 cell lines are confirmed by western blot analysis (B), SNU-638 cell line is significantly reduced CEACAM1 expression level.

TRC control: transfected with control vector, shCEACAM1: transfected shRNA of CEACAM1, given number was each clone.

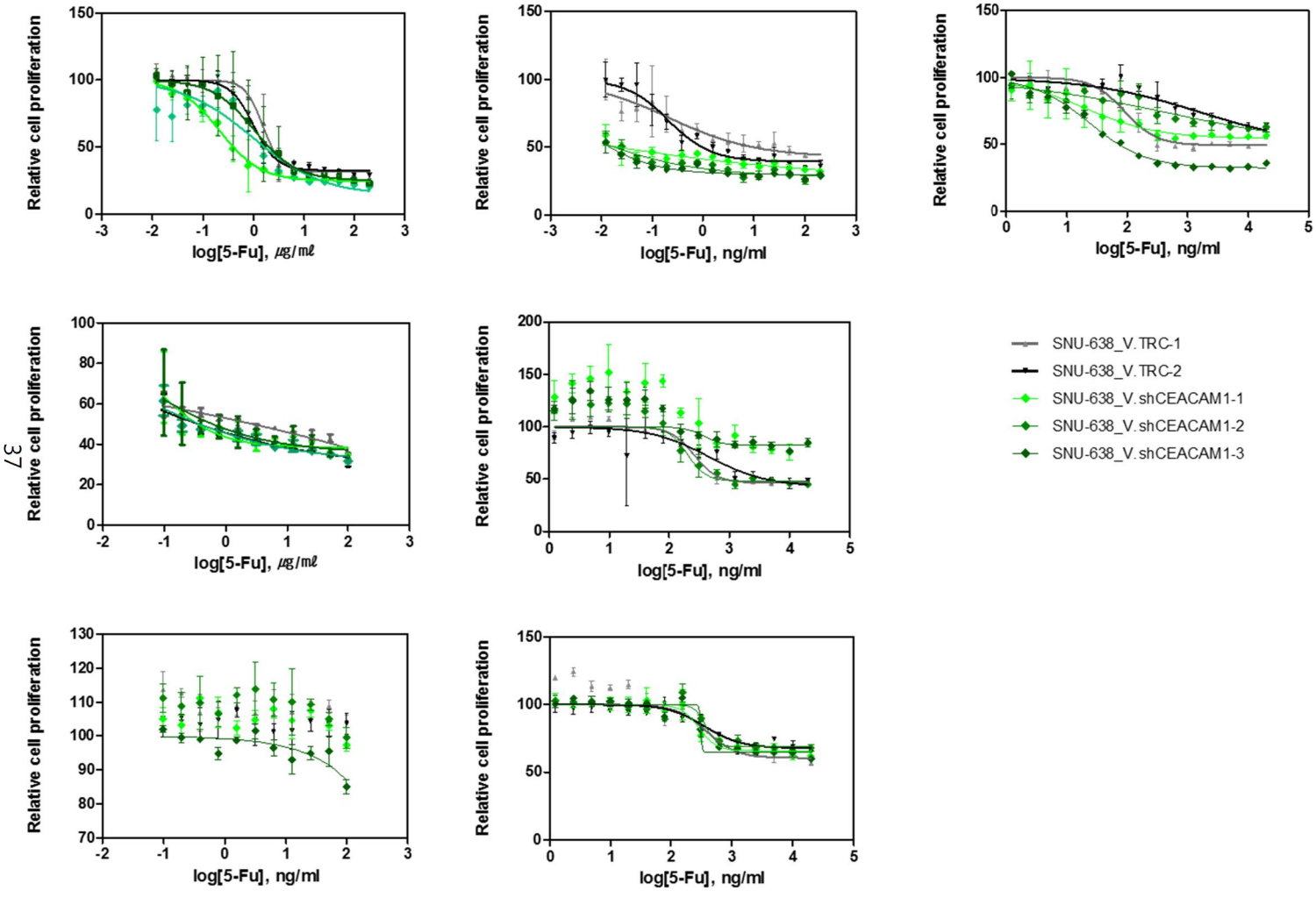
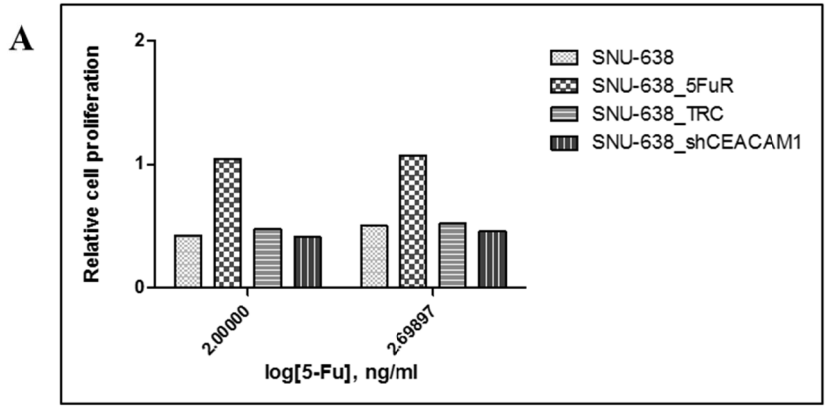

Relative cell proliferation
log[5-Fu], ㎍/㎖
log[5-Fu], ng/ml
SNU-638_V.TRC-1
SNU-638_V.TRC-2
SNU-638_V.shCEACAM1-1
SNU-638_V.shCEACAM1-2
SNU-638_V.shCEACAM1-3

Figure 5. Expression of CEACAM1 is not correlated with cell proliferation rate of 5-Fu.

I performed cell proliferation as treated with serial diluted 5-Fu at multiple times, however, there is less correlation between CEACAM1 expression level and cell proliferation rate.

SNU-638_V.TRC was transfected TRC control vector, and SNU-638_V.shCEACAM1 was transfected shRNA vector. Each of the data are represented as mean ± SD three independent experiments.


A
Relative cell proliferation
2
1
0
SNU-638
SNU-638_5FuR
SNU-638_TRC
SNU-638_shCEACAM1
2.00000
2.69897
log[5-Fu], ng/ml

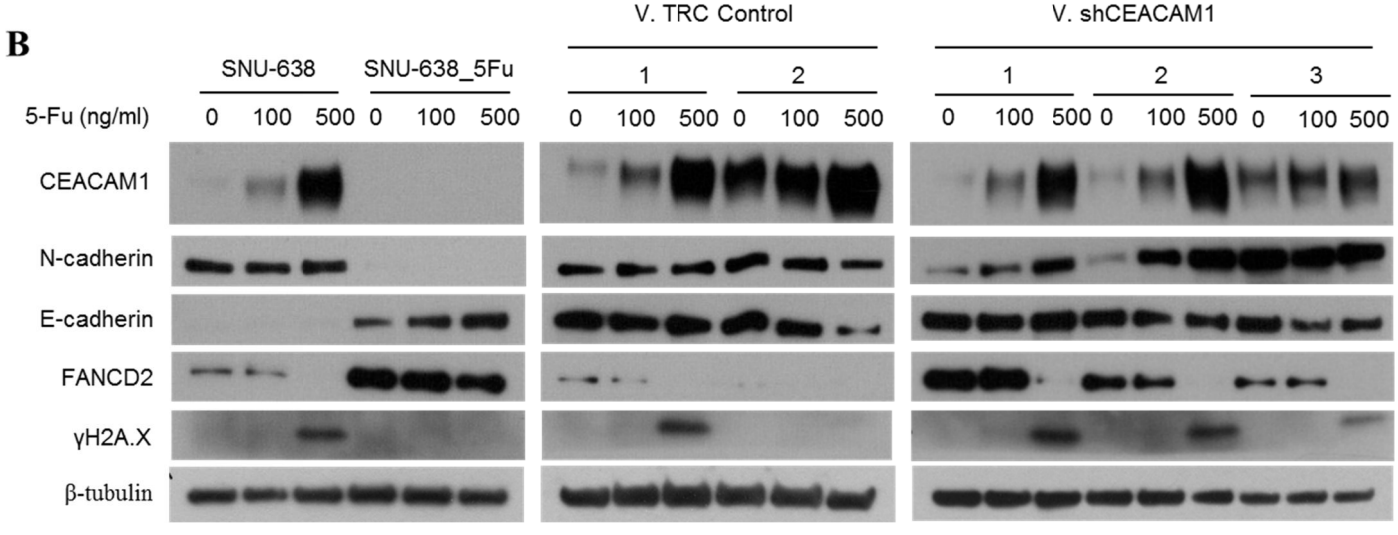
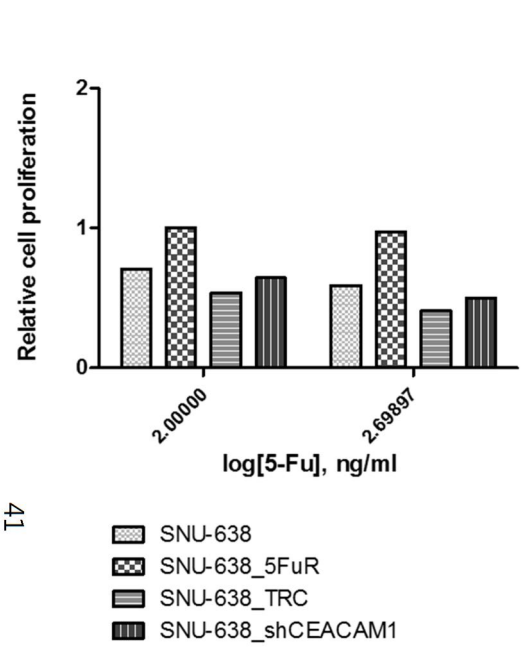

B
SNU-638
SNU-638_5Fu
V. TRC Control
V. shCEACAM1
1
2
1
2
3
5-Fu (ng/ml)
0 100 500 0 100 500 0 100 500 0 100 500 0 100 500 0 100 500 0 100 500
CEACAM1
N-cadherin
E-cadherin
FANCD2
γH2A.X
β-tubulin

Figure 6. Expression of CEACAM1 is not correlated with cadherin that mediated cell adhesion.

As treated with 100, and 500 ng/mL 5-Fu, I performed cell proliferation assay (A) and western blot analysis (B) in SNU-638 parental, 5-Fu resistant, TRC control, and shCEACAM1 cell lines.


Relative cell proliferation
2
1
0
2.00000
2.69897
log[5-Fu], ng/ml
SNU-638
SNU-638_5FuR
SNU-638_TRC
SNU-638_shCEACAM1

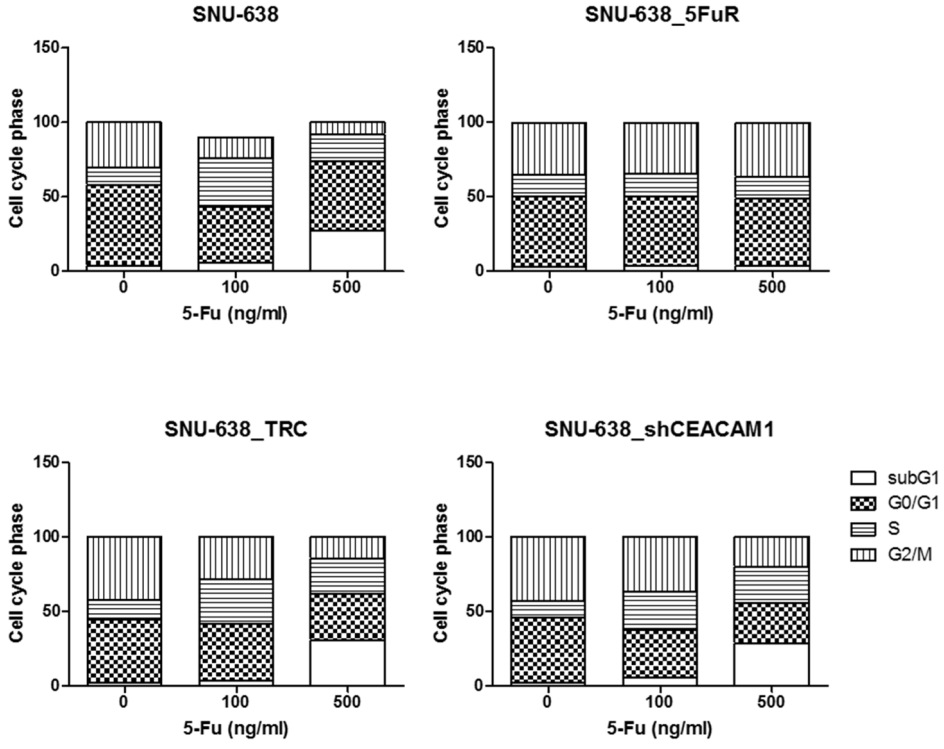

SNU-638
SNU-638_5FuR
SNU-638_TRC
SNU-638_shCEACAM1
Cell cycle phase
150
100
50
0
0
100
500
5-Fu (ng/ml)
subG1
G0/G1
S
G2/M

Figure 7. Expression of CEACAM1 is not influenced to cell cycle.

As treated with 100 and 500 ng/mL 5-Fu, I performed cell proliferation assay (A) and cell cycle analysis (B-E). DNA staining using PI solution, and analyzed by FACS. SNU-638_TRC (D) means SNU-638_TRC control, SNU-638_shCEACAM1 (E) means SNU-638_shCEACAM1.

Table 4. Numeric representative cell cycle. It matched to Figure 7.

SNU-638					
	%			Relative Rate to control(%)	
5-Fu (ng/ml)	0	100	500	100	500
subG1	3.8	5.9	27	0.55	6.11
G0/G1	54.4	37.5	46.9	-0.31	-0.14
S	11.9	32.5	18.1	1.73	0.52
G2/M	30	24.1	8.1	-0.20	-0.73

SNU-638_5FuR					
	%			Relative Rate to control(%)	
5-Fu (ng/ml)	0	100	500	100	500
subG1	3.3	3.6	3.9	0.09	0.18
G0/G1	47	46.4	45	-0.01	-0.04
S	14.6	15.7	14.6	0.08	0.00
G2/M	35	34.2	36.4	-0.02	0.04

SNU-638_TRC					
	%			Relative Rate to control(%)	
5-Fu (ng/ml)	0	100	500	100	500
subG1	2.1	4	30.9	0.90	13.71
G0/G1	42.3	37.8	31.2	-0.11	-0.26
S	13.8	29.7	23.7	1.15	0.72
G2/M	41.8	28.5	14.2	-0.32	-0.66

SNU-638_shCEACAM1					
	%			Relative Rate to control(%)	
5-Fu (ng/ml)	0	100	500	100	500
subG1	2.1	6.1	28.9	1.90	12.76
G0/G1	43.8	31.3	26.8	-0.29	-0.39
S	11.6	26.4	24.6	1.28	1.12
G2/M	42.5	36.2	19.7	-0.15	-0.54

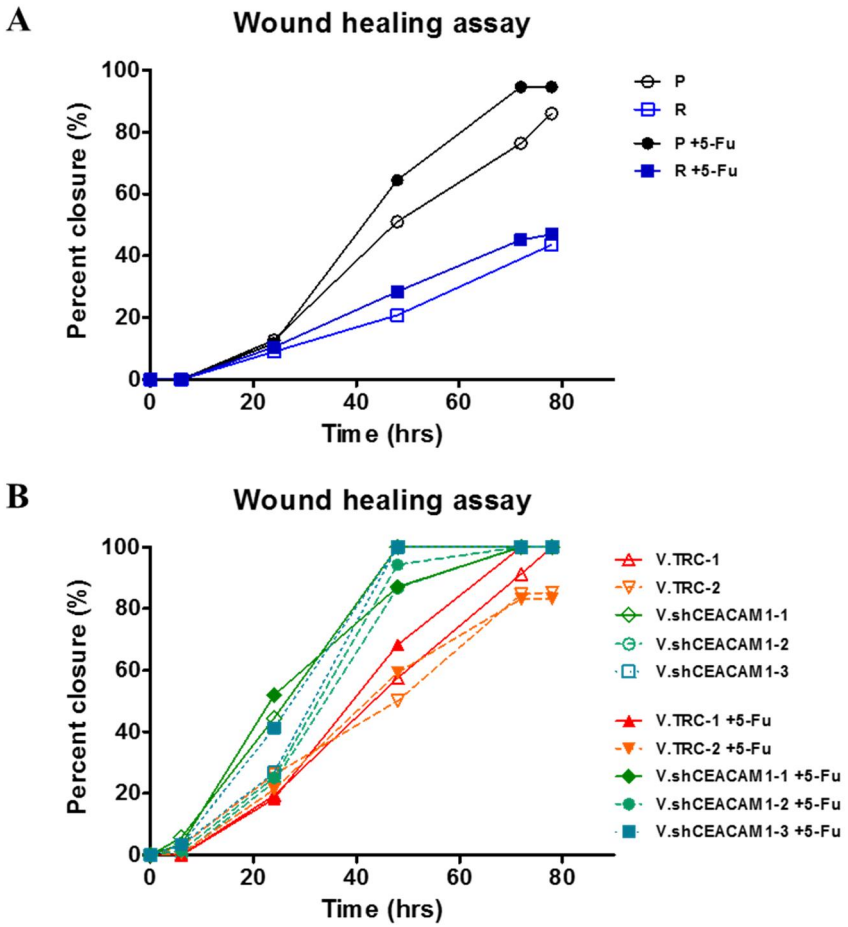


Figure 8. Expression of CEACAM1 is influenced to wound healing capacity.

A is represented difference in wound healing ability between parental, and 5-Fu resistant cell line, and B is TRC, shCEACAM1 cell lines. Open figures represented each cell lines, and closed figures are 100 ng/mL of 5-Fu treated cell lines.

CEACAM1 expression was increased by treatment of chemotherapeutic agents and irradiation

Then, I speculated that if increased CEACAM1 was the phenomenon of cell death, it was also increased by other cell death stimuli. For verification, I treated 10 ng/mL of 5-Fu, that was low dose for minimalized 5-Fu effect, and 1 μM of oxaliplatin and irinotecan. As shown in Figure 9A and B, represented increased CEACAM1 not only by 5-Fu but also by sole or combination treated with oxaliplatin and irinotecan in parental gastrointestinal cancer cell lines. Then, I irradiated 4 Gy to SNU-638, SNU-C5 cell lines; CEACAM1 was increased as similar level which 5-Fu treatment (Figure 9C). Ten and 20 Gy of radiation also influenced to CEACAM1 expression level, however, there was slightly difference between 10, and 20 Gy of radiation (Figure 9D).

Through these results, I suggested that increased CEACAM1 expression was the result of phenomenon of cell death induced by chemotherapeutic agents and radiation.

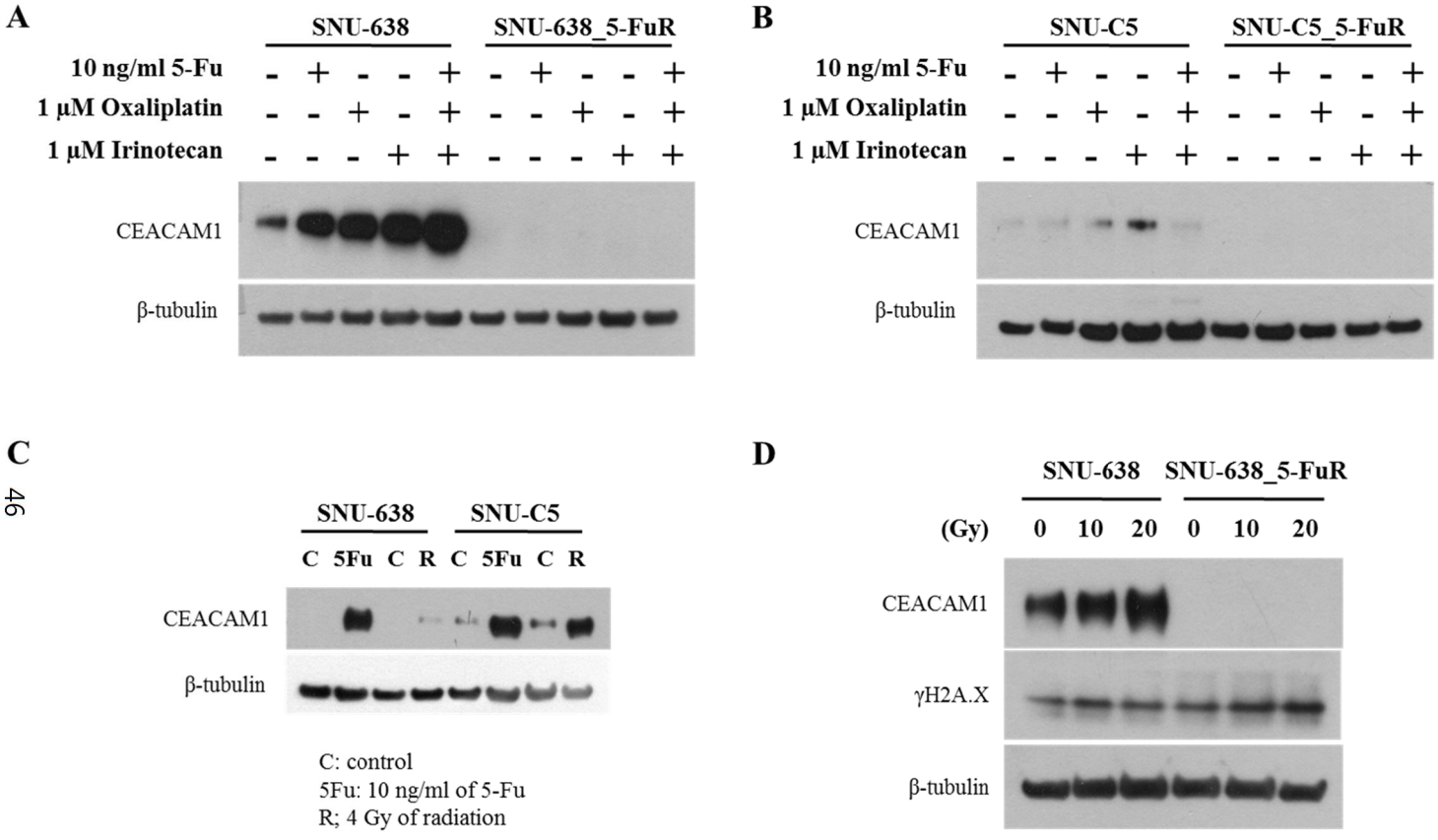

A
SNU-638
SNU-638_5-FuR
10 ng/ml 5-Fu
1 μM Oxaliplatin
1 μM Irinotecan
CEACAM1
β-tubulin
B
SNU-C5
SNU-C5_5-FuR
10 ng/ml 5-Fu
1 μM Oxaliplatin
1 μM Irinotecan
CEACAM1
β-tubulin
C
SNU-638
SNU-C5
C 5Fu C R C 5Fu C R
CEACAM1
β-tubulin
C: control
5Fu: 10 ng/ml of 5-Fu
R; 4 Gy of radiation
D
SNU-638
SNU-638_5-FuR
(Gy) 0 10 20 0 10 20
CEACAM1
γH2A.X
β-tubulin

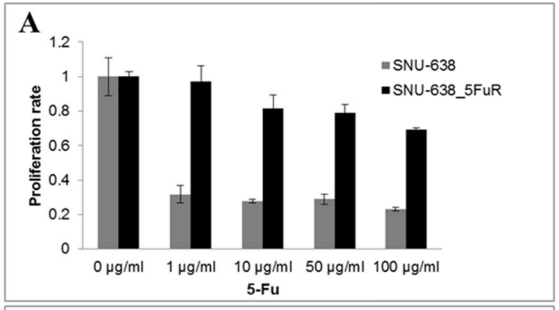
Figure 9. Expression of CEACAM1 is induced by 5-Fu, oxaliplatin, irinotecan, and radiation.

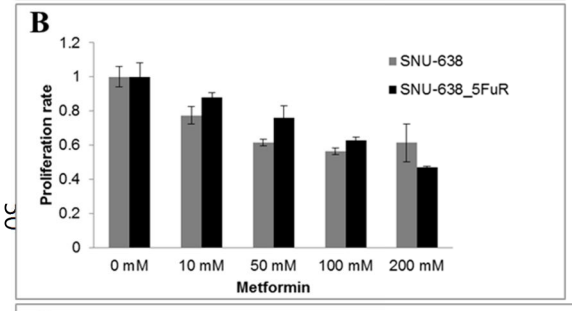
10 ng/mL of 5-Fu, 1 μM of oxaliplatin and irinotecan are treated to gastric cancer cells (A), or colorectal cancer cells (B) alone, or combination. Irradiation increases CEACAM1 expression level, as 4 Gy of radiation to gastrointestinal cell lines (C), and dose-dependent treatment of radiation (D). CEACAM1 expression level is confirmed by western blot analysis.

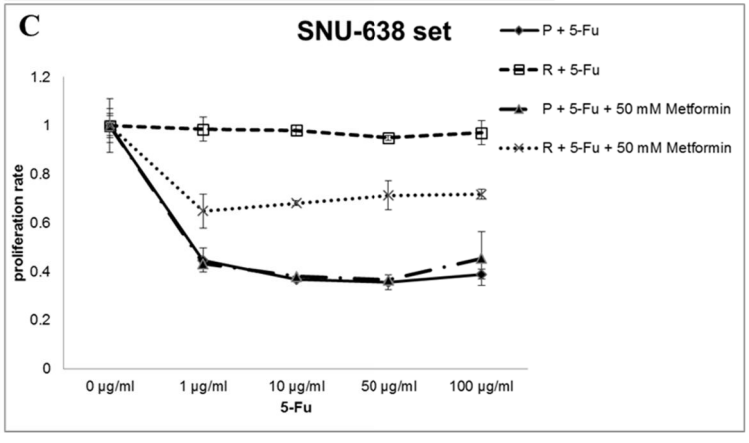
Metformin inhibits cell proliferation and increased CEACAM1 expression

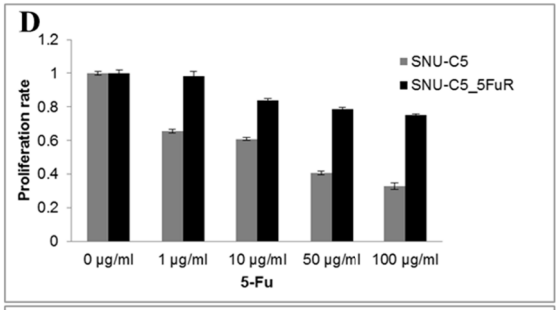
Besides CEACAM1 function in cell adhesion and cell death, CEACAM1 regulates insulin clearance and insulin resistance *in vivo* [43]. Thus, I wonder that whether CEACAM1 related with insulin sensitivity regulating agents, metformin. Since metformin has anticancer effect, I performed cell proliferation assay. As treated with 1, 10, 50, and 100 μg/mL of 5-Fu, cell proliferation rate of parental cell lines was decreased over 60%, and only 20% or less in 5-Fu resistant cell lines (Figure 10A, D), which data was replicable the above results. In the case of metformin, parental and 5-Fu resistant cell lines had similar response; as treated with 10, 50, 100, and 200 mM of metformin, cell proliferation rate was decreased depend on metformin concentration in both cell lines (Figure 10B, E). Interestingly, as combination treatment of serial diluted 5-Fu with 50 mM of metformin, there was synergistic effect in 5-Fu resistant cell lines (Figure 10C, F), not in parental cell lines. This results suggested that 5-Fu resistant gastrointestinal cancer cell lines were more sensitive to metformin. For verified metformin activation pathway as well CEACAM1 expression level in gastrointestinal cancer

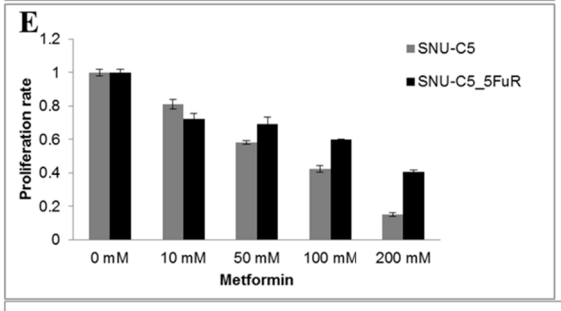
cell lines as treated with metformin, I confirm the protein expression level. As shown in Figure 11, metformin inhibits mTOR concomitant with activated AMPKα in parental-, 5-Fu resistant cell lines. And CEACAM1 expression level was increased as treated with 5-Fu, only in parental cell lines. It was suggested that induced CEACAM1 was correlated with 5-Fu induced cell death.


A
Proliferation rate
1.2
1
0.8
0.6
0.4
0.2
0
SNU-638
SNU-638_5FuR
0 μg/ml
1 μg/ml
10 μg/ml
50 μg/ml
100 μg/ml
5-Fu


B
Proliferation rate
1.2
1
0.8
0.6
0.4
0.2
0
SNU-638
SNU-638_5FuR
0 mM
10 mM
50 mM
100 mM
200 mM
Metformin


C
SNU-638 set
proliferation rate
1.2
1
0.8
0.6
0.4
0.2
0
P + 5-Fu
R + 5-Fu
P + 5-Fu + 50 mM Metformin
R + 5-Fu + 50 mM Metformin
0 μg/ml
1 μg/ml
10 μg/ml
50 μg/ml
100 μg/ml
5-Fu


D
Proliferation rate
1.2
1
0.8
0.6
0.4
0.2
0
SNU-C5
SNU-C5_5FuR
0 μg/ml
1 μg/ml
10 μg/ml
50 μg/ml
100 μg/ml
5-Fu


E
Proliferation rate
1.2
1
0.8
0.6
0.4
0.2
0
SNU-C5
SNU-C5_5FuR
0 mM
10 mM
50 mM
100 mM
200 mM
Metformin

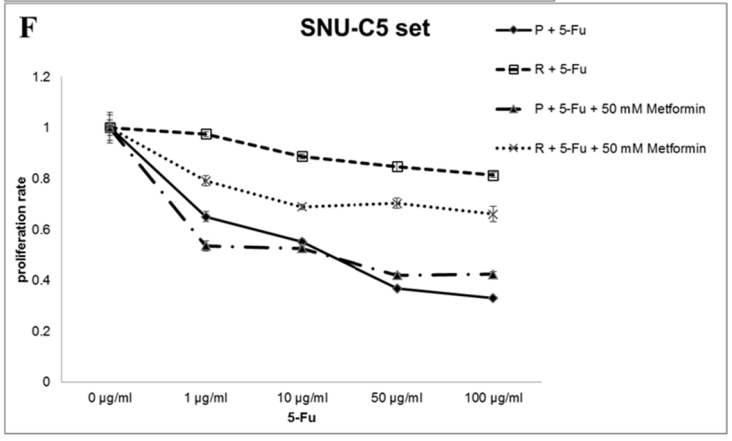
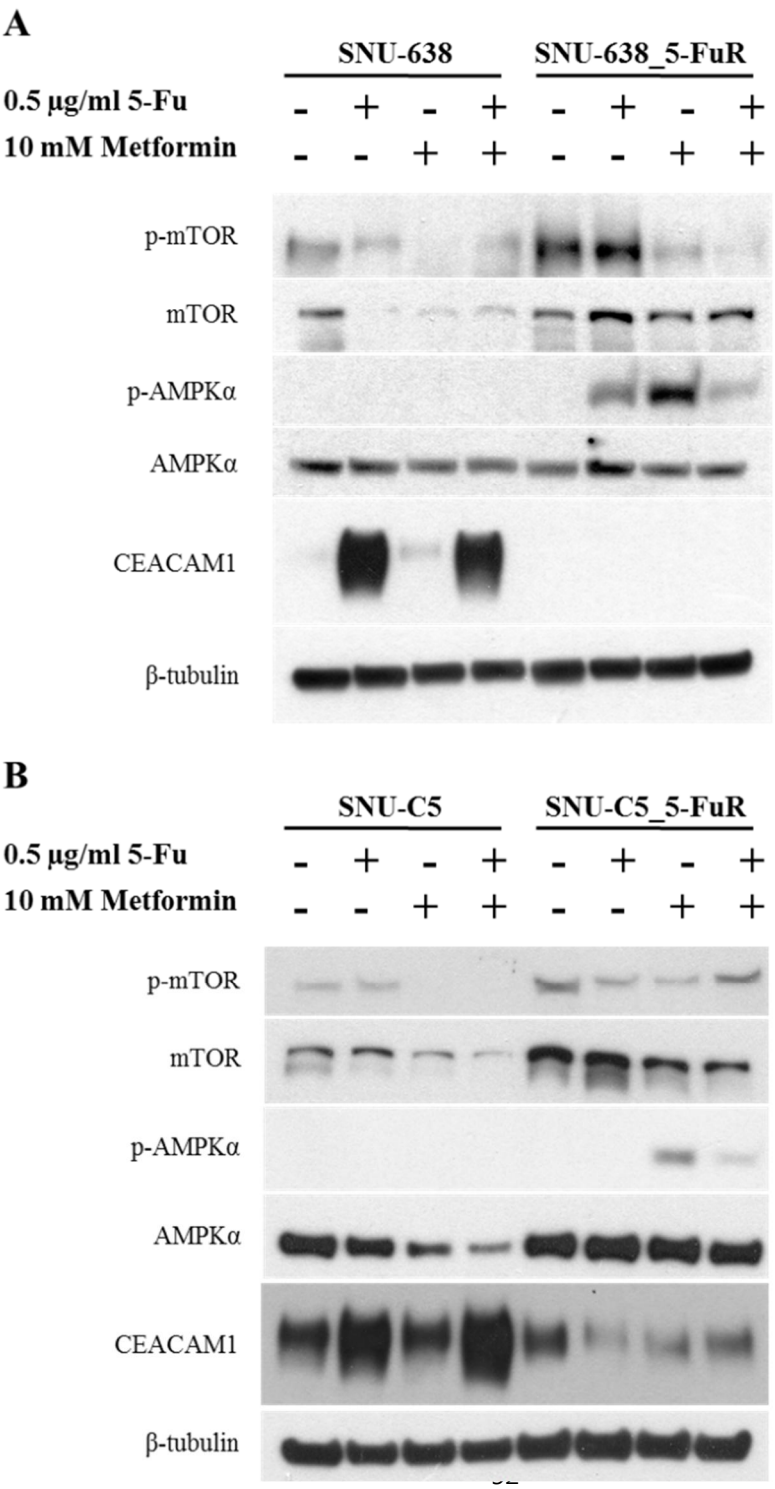

F
SNU-C5 set
proliferation rate
1.2
1
0.8
0.6
0.4
0.2
0
P + 5-Fu
R + 5-Fu
P + 5-Fu + 50 mM Metformin
R + 5-Fu + 50 mM Metformin
0 μg/ml
1 μg/ml
10 μg/ml
50 μg/ml
100 μg/ml
5-Fu

Figure 10. Metformin has synergistic effect with 5-Fu.

A and D, relative cell proliferation as treated with 5-Fu 1, 10, 50, and 100 μg/mL, B and E represents as treated with 10, 50, 100, and 200 mM of metformin, C and E represents combination treatment of various dose of 5-Fu with 50 mM of metformin. Proliferation assay is performed with SNU-638 (A-C), and SNU-C5 (D-F). All the data are represented as mean ± SD three independent experiments.

Figure 11. Metformin regulates AMPK-mTOR axis pathway by phosphorylation.

I perform the western blot analysis of AMPK, mTOR and their phosphorylation form whether metformin effected to gastrointestinal cancer cell lines. Also, confirm the CEACAM1 expression level. A, in SNU-638 and SNU-638_5FuR, B, in SNU-C5 and SNU-C5_5FuR cell lines. (Next page)


A
SNU-638
SNU-638_5-FuR
0.5 μg/ml 5-Fu
10 mM Metformin
p-mTOR
mTOR
p-AMPKα
AMPKα
CEACAM1
β-tubulin
B
SNU-C5
SNU-C5_5-FuR
0.5 μg/ml 5-Fu
10 mM Metformin
p-mTOR
mTOR
p-AMPKα
AMPKα
CEACAM1
β-tubulin

Discussion

CEACAM1 is a sort of cell surface molecules belongs to immunoglobulin superfamily, that involved in various cellular functions; cell growth, differentiation, role in insulin homeostasis, neo-angiogenesis, apoptotic initiation, and immune modulation [35, 38, 44]. In some tumors, CEACAM1 was considered as a tumor suppressor, since the expression level was deceased in breast, colon, and prostate cancer [37, 44-46]. However, there are controversial observations that CEACAM1 expression was upregulated in thyroid, gastric, and lung cancers [37, 44]. In addition, CEACAM1 related with sphere formation in 3-demensional (D) culture system, and 5-Fu chemo-sensitivity [47].

In this study, I investigated CEACAM1 expression level in gastrointestinal cancer cell lines and in 5-Fu resistant cell lines. Through this, ultimately I intended suggestion about overcome of 5-Fu resistance.

CEACAM1 was expressed in most of gastrointestinal cancer cell lines (Figure 1, 2). From among these cell lines, I notified SNU-638, SNU-C5 and their 5-Fu resistant cell lines that had different CEACAM1

expression pattern between parental and 5-Fu resistant cell lines. In the parental cell lines, SNU-638 and SNU-C5, CEACAM1 was highly expressed, while low expressed in 5-Fu resistant cell lines. To investigate whether CEACAM1 correlation with 5-Fu, I used these two cell line sets.

Parental gastrointestinal cancer cell lines were more sensitive to 5-Fu than 5-Fu resistant cell lines (Figure 3). As treated with 5-Fu, CEACAM1 was increased by dose-, and time-dependent manner only in parental cell lines. At the same time, cell proliferation rate was decreased and apoptotic proteins such as cleaved PARP and caspase-3 were increased. It was suggested that 5-Fu sensitivity were correlated with CEACAM1 expression level. Then, I hypothesized that CEACAM1-mediated 5-Fu induced cell death in parental cell lines. CEACAM1 implicated in initiating of apoptotic signals that through loss of CEACAM1 expression, which proposed generated hyperplasia, oncogenic mutation, and inactivation of APC [36, 45]. Hence I suggested that 5-Fu induced CEACAM1-mediated cell death in gastrointestinal cell lines. To verify this, I constructed CEACAM1 knockdown cell lines. Designed shRNA was targeted whole form of CEACAM1. Despite of the

controversy, CEACAM1 may acts as tumor suppressor [37, 44-46], thus I predicted that CEACAM1 knockdown cell lines were affected to cell proliferation itself. Also, I hypothesized that 5-Fu induced cell death via CEACAM1-mediated signaling pathway, if CEACAM1 was knockdown, gastrointestinal cancer cells were less sensitive to 5-Fu. To verify this, I performed cell proliferation assay, cell cycle, and confirm the protein expression, however, there were no significant changes between parental cell lines and knockdown cell lines. Multiple times repeated cell proliferation assays were inconsistent, it reflect that no correlation between cell proliferation rate and expression level of CEACAM1 (Figure 5).

5-Fu was sensitized cancer cells to double stranded DNA breaks (DSBs) by inhibition of homologous recombination repair [48]. Through this, I speculated that CEACAM1 knockdown cell lines had similar gene expression tendency and cell cycle distribution with 5-Fu resistant cell lines which influenced to 5-Fu, however, that was fallacious hypothesis (Figure 6, 7). Response such as gene expression and cell proliferation to 5-Fu of CEACAM1 knockdown cell lines had more similarities with parental gastrointestinal cancer cell lines. According to above results, I

suggested that increased CEACAM1 expression as treated with 5-Fu was not CEACAM1-mediated cell death but other mechanisms.

Then I speculated that CEACAM1 increased by 5-Fu was the phenomenon of cell death, if there was correlation, CEACAM1 was also increased by other cell death stimuli. There are therapeutic strategies to improve anticancer effect of 5-Fu and reduce recurrence rate; adjuvant chemotherapy with oxaliplatin and irinotecan in combination with 5-Fu or radiotherapy were performed [49, 50]. These chemotherapeutic agents and radiation also induced CEACAM1 expression level in parental cell lines not in 5-Fu resistant cell lines (Figure 9). Thus, I suggested that 5-Fu induced CEACAM1 was related to cell death. And increased CEACAM1 was a characteristic of parental gastric intestinal cancer cell lines.

Finally, I investigated CEACAM1 expression level as treated metformin, one of the insulin sensitivity regulating agent. CEACAM1 originally identified as a substrate of the insulin receptor tyrosine kinase in rat [51], and it is regulates of insulin resistance [43]. Insulin resistance causes type II diabetics, and diabetic patients has increased incidence rate of various cancer [52, 53]; I wonder that cellular response to

diabetic therapeutic agent, metformin. Since discovered in the 1920s, metformin is widely used type II diabetic therapeutic agents with less side effects [54]. Besides glucose lowering, and insulin sensitized effects, metformin has chemoprevention and anticancer effect [54, 55]. With these basis, I performed cell proliferation assay that metformin treatment combination with 5-Fu. As shown in Figure 10, gastrointestinal cancer cell lines affected to metformin, cell proliferation rate was decreased as dose-dependent manner of metformin. And combination treatment of 5-Fu with metformin had synergistic effect especially in 5-Fu resistant cancer cell lines. The response of metformin was through activation of AMPKα and inhibition of mTOR axis, 5-Fu resistant cell lines were more affected these signaling pathway (Figure 11). Metformin was correlated with cell proliferation rate, however, does not induces CEACAM1 expression level itself in gastrointestinal cancer cell lines. In accordance with suggestion that CEACAM1 as cell death related protein, I predicted CEACAM1 induction was concomitant treated with metformin. Two lines of explanation about distinction among hypothesis and results. First, metformin dose that I used affected to cell signaling pathway, but not sufficient to induce cell

damage and CEACAM1 expression level. Second, because of 5-Fu and metformin has different working pathway, CEACAM1 was not induced by treatment of metformin.

In summary, CEACAM1 was increased by chemotherapeutic agents, 5-Fu, oxaliplatin, and irinotecan, and radiation, not other proliferation inhibitors such as metformin in gastrointestinal cancer cell lines. Thus I suggested that CEACAM1 could be used as an indicator of effect for chemotherapeutic agents, especially 5-Fu.

PART Ⅱ. Metformin increases chemo-sensitivity via gene downregulation encoding DNA replication proteins in 5-Fu resistant colorectal cancer cells

Abstract

Metformin is most widely prescribed for type 2 diabetics. Recently, there evidence has shown that metformin has anticancer effects on pancreatic-, colorectal-, ovarian-, and other cancers. Because metformin has less adverse effects and is inexpensive, it could be a useful chemo-therapeutic agent with anticancer effects. In this study, I demonstrated metformin inhibited by cell proliferation, cell migration ability, clonogenic ability, and cancer stem cell population. Metformin also induced cell cycle arrest in parental-(SNU-C5), and 5-Fu resistant-colorectal cancer cell line (SNU-C5_5FuR). Moreover, a treatment that combines 5-Fu and metformin was found to have a synergistic effect on the cell proliferation rate, especially in SNU-C5_5FuR, which were mediated by the activation of AMPK pathway and NF-κB pathway, well-known metformin mechanisms. In this study, I suggested novel anticancer mechanism of metformin that inhibited DNA replication machinery, such as the MCM family in SNU-C5_5FuR. In

conclusion, I provided that how metformin acts as not only a chemo-sensitizer, but also as a synergistic effector of 5-Fu in the 5-Fu resistant-cell line. I speculate that metformin used for adjuvant therapy is effective on 5-Fu resistant cancer cells.

Key words: colorectal cancer, 5-Fu resistant-cell line, metformin, cancer stem cell (CSC), MCM family, DNA replication

Introduction

Colorectal cancer is the third most diagnosed cancer with an annual estimated death of 60,000 [56]. 5-Fluorouracil (5-Fu) is the standard chemotherapeutic agent in colorectal cancer that acts as an antimetabolite drug through thymidylate synthase (TS) inhibition and incorporated into nucleic acid, DNA, and RNA. Despite the usage frequency, 5-Fu has low effectiveness in colorectal cancer at about 10 to 15% [22]. Recently, combination therapies with other drugs like irinotecan and oxaliplatin are suggested to improve 5-Fu effectiveness using the independent activity pathway. Nevertheless, 5-Fu still has low effectiveness due to drug resistance. Furthermore, there are diverse mechanisms related to 5-Fu resistance like changes in drug uptake and/or catalytic enzyme activities [57, 58].

Metformin (N',N'-dimethylbiguanide) was developed as a type 2 diabetic therapeutic agent [59]. Unlike the other biguanides, buformin

and phenformin, metformin is the most frequently prescribed drug due to minimized toxicity and side effects [59, 60]. Interestingly, there are some studies that state metformin is related to anticancer effects: metformin significantly decreased the incidence risk of pancreatic-, colorectal-, and ovarian cancers [55, 61-63], as well as various cancer cell lines [64-68]. Furthermore, metformin influences apoptosis and cell cycle arrest, which reduces cancer cell populations [69, 70]. The mechanism of metformin action is well-studied by working through pivotal AMPK/mTOR pathways [71]. Metformin activates AMPK after LKB1 and sequentially inactivates mTOR. Along this pathway, p53 is activated according to autophagy and decreased protein synthesis. The cell cycle is arrested as a result, which means that metformin mechanism is related to the chemotherapeutic effect. Moreover, in SW620 colon cancer cell line, metformin affects cell proliferation, apoptosis, and cell cycle via selectively targeted CD133+ cancer stem cell populations [72].

In this paper, I showed metformin selectively affecting cell proliferation and metastatic behavior on 5-Fu resistant-colorectal cancer cell lines caused by the inhibition of DNA replication machinery.

Materials and methods

Cell culture and Chemicals

SNU-C5, a human colorectal cancer cell line, was obtained from the Korean Cell Line Bank. SNU-C5_5FuR, a 5-fluorouracil-resistant population of SNU-C5, was established as previously described [40, 41] by treatment with 4000 ng/mL of 5-Fu (Sigma- Aldrich. Cell lines were cultured in RPMI1640 (Thermo-Fisher scientific) medium supplemented with 10% fetal bovine serum and 1.1% penicillin/streptomycin.

Metformin and 5-Fu were purchased from Sigma-Aldrich and were dissolved in deionized water and DMSO, respectively, and before being stored at 4°C protected from light.

Cell proliferation, Migration, and Clonogenic assay

The cell proliferation rate was assessed by NADH-dehydrogenase in the live cell using Ez-Cytox (Daeillab service, Seoul, Korea). 50,000

cells/well were seeded in a 96-well cell culture plate and incubated for 16 h before being treated by the 5-Fu and/or metformin. Then, I added 10 μl of Ez-Cytox solution to each well. The absorbance at 450 nm was measured by a MULTISKAN FC Microplate Photometer (Thermo-Fisher Scientific). The assay was performed three times while.

50,000 cells/well were seeded on a 24-well plate with wound-healing insert (Cell biolabs) and incubate for overnight for a cell migration assay. The insert was carefully removed and incubated with cell culture medium containing 5-Fu and/or metformin. 400 × magnification images were taken at the initial wound and after 6, 24, 48, and 72 h using a CCD camera (Olympus).

For the clonogenic assay, 50,000 cells/well were seeded on a 6-well plate. After incubation overnight, cells were exposed to 5-Fu and/or metformin for 72 h, then trypsinized cells were re-seeded on a 60-mm culture dish with 0.5×10^3 cells. The cells were then incubated for 14 days in humidified incubators. Colonies were fixated with methanol and acetic acid in a 1:7 solution for 10 min at RT and staining with 0.5% crystal violet for 1 h at RT. The colonies were counted after a water wash and air drying.

Tumor sphere formation was performed using reduced growth factor (RGF)-basement membrane extract (BME) (Thermo-Fisher scientific), with 50,000 cells/well of cells on a 24-well plate, then treated with 5-Fu and/or metformin.

Apoptotic analysis

To detect apoptotic cell death, I used an Annexin V-FITC/PE staining kit (Invitrogen,) according to manufacturer's procedure and cell harvested after 5-Fu and metformin treatment. Then, cells were resuspended to 100 μl per sample of 1X Annexin-binding buffer with 5 μl of Annexin V-FITC, and 1 μl of 100 μg/mL propidium iodide (PI) (Sigma-Aldrich) solution. After incubation at RT for 15 min, the cells were analyzed by flow cytometry (FACS CantoII ™).

Cell cycle analysis

To analyze the cell cycle distribution, cells were fixed overnight in 70% ethanol at −20°C. The cellular DNA was stained with 100 μg/mL of PI for 30 min on ice and analyzed using a flow cytometry based on DNA content to determine the percentage of cells in different cell phases and apoptosis.

Reverse transcriptase (RT) – PCR and quantitative real-time RT-PCR

cDNA was synthesized from 2 μg of total RNA isolated by using easy-BLUE™ kits (Intron biotechnology) and a Quantitect Reverse transcription kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The PCR mixture contained 1 μl of 100 ng/μl cDNA, 10× buffer, 2.5 mM of dNTP, 0.1 pM of primers and 1 unit of Taq DNA polymerase (Intron biotechnology) for a total of 15 μl. The following PCR conditions were used: initial denaturation for 5 min at 94° C, cycling at 94° C (30 sec), 55° C (30 sec), and 72° C (30 sec) for 35 cycles, and final elongation for 7 min at 72° C. PCR was performed on a thermal cycler (PCR System 9700, Applied Biosystems). The quantitative RT-PCR was performed using 2X SYBR on a 7300 instrument (Applied Biosystems) with the following conditions: 2 min at 50° C, 10 min at 95° C, and 40 cycles of 15 s at 95° C, and 1 min at

60° C. Each reaction was performed three times on a 96-well plate. Raw Ct was calculated with 7300 system version 1.4.0. The used oligonucleotide primer sequences were as follow [73, 74]:

CD133 S: 5' -CTGGGGCTGCTGTTTATTATTCTG-3' ,

CD133 AS: 5' -ACGCCTTGTCCTTGGTAGTGTTG-3'

CD44 S: 5' -GTGATCAACAGTGGCAATGG-3'

CD44 AS: 5' - GGGCCCTAATTTCAGAAAGC-3'

β-actin S: 5' -GAGACCTTCAACACCCCAGC-3'

β-actin AS: 5' -GCTCATTGCCAATGGTGATG-3'

Western blot analysis

Whole cell lysates were prepared by RIPA kits (Atto, Tokyo, Japan) while the protein concentration was accomplished by a SMART^{TM} micro BCA kit (Intron biotechnology). For immunoblot analysis, 20 μg of protein and 4 × SDS sample buffer mixture were boiled at 95°C and loaded on 4-12% Bis-Tris pre-cast gel (Thermo-Fisher scientific). Separated proteins were transferred onto PVDF membrane (Bio-Rad, CA, USA), which were blocked with 2.5% non-fat dry milk and 0.5% Tween 20-TBS buffer containing 1mM of $MgCl_2$. Primary antibodies

against PARP (BD,)(1:1000), caspase-3, CD133, VEGF, Chk1, p-Chk1, Chk2, p-Chk2, PCNA, MCM2, p-ATM (Abcam, Cambridge, UK)(1:1000), mTOR, p-mTOR, AMPKα, p-AMPKα (Cell signaling, MA, USA), HIF-1α (Sigma-Aldrich), β-actin, and β-tubulin (Santa Cruz, CA, USA) (1:5000) were incubated overnight at 4°C. Peroxidase conjugated secondary antibody were against rabbit or mouse (Jackson Immunoreasearch) diluted 1:5000 and incubated for 1 h at RT. To detect the protein signals, WEST-ZOL™ (Intron biotechnology) was used as a chemiluminescent solution. Finally, the membrane was exposed to Fuji RX film for 1-10 min.

Immunophenotyping

FcR blocking reagents and CD133-PE antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to cells and incubated for 10 min at 4°C. The CD133 expression was detected by FACS.

Statistical analysis

For statistical analysis, GraphPad Prism 5.03 was used. The mean ± SD was determined for experiments.

RNA Sequencing

- mRNA Sequencing

The total RNA was isolated from cell lysate using Trizol and RNeasy Kit (Qiagen) according to manufacturer's protocol. RNA purity was determined by assaying 1 μl of total RNA extract on a NanoDrop8000 spectrophotometer. Total RNA integrity was checked using an Agilent Technologies 2100 Bioanalyzer with an RNA Integrity Number (RIN) value.

mRNA sequencing libraries were prepared according to the manufacturer's instructions (Illumina Truseq stranded mRNA library prep kit). mRNA was purified and fragmented from total RNA (0.8 μg) using poly-T oligo-attached magnetic beads using two rounds of purification. Cleaved RNA Fragments primed with random hexamers were reverse transcribed into first strand cDNA using reverse transcriptase, random primers, dUTP in place of dTTP. (The incorporation of dUTP quenches the second strand during amplification, because the polymerase does not incorporate past this nucleotide.) These cDNA fragments then had the

addition of a single 'A' base and subsequent ligation of the adapter. The products were purified and enriched with PCR to create the final strand specific cDNA library. The quality of the amplified libraries was verified by capillary electrophoresis (Bioanalyzer, Agilent).

After qRT-PCR using SYBR Green PCR Master Mix (Applied Biosystems), I combined libraries that index tagged in equimolar amounts in the pool. Cluster generation occurred in the flow cell on the cBot automated cluster generation system (illumina). And then the flow cell loaded on HISEQ 2500 sequencing system (Illumina), performed sequencing with 2x100bp read length.

- RNA-Seq analysis method

Mapping reads on a reference genome and calculating expression between samples

At first, reads for each sample were mapped to the reference genome (human hg19) [75] by Tophat (v2.0.13) [76]. The aligned results were added to Cuffdiff (v2.2.0) [77] to report differentially expressed genes. For library normalization and dispersion estimation, geometric and blind

(“blind” when each condition has single replicates, or “pooled” when multiple replicates (more than one sample) are available) methods [77] were applied.

Identification of DEGs and functional enrichment analysis

Cuffdiff provides various output files, and using one of its outputs, “gene_exp.diff”, DEGs (Differentially Expressed Genes) were identified. To detect DEGs between sample1 as control and sample2 as case, two filtering processes were applied. First, using Cuffdiff status code, genes that only have “OK” status were extracted. Status code indicates whether each condition contains enough reads in a locus for a reliable calculation of expression level, and “OK” status means the test is successful to calculate gene expression level. For the second filtering, 2 fold change was calculated and only genes belonging to the following range were selected.

$$\text{Up-regulated: } \log2[\text{case}] - \log2[\text{control}] > \log2(2) = 1$$

$$\text{Down-regulated: } \log2[\text{case}] - \log2[\text{control}] < \log2(1/2) = -1$$

For ontology analysis, genes after 2 fold change were picked and applied to DAVID [78] as an input to get a comprehensive set of functional annotation. Disease, Gene ontology, pathway categories were selected, and Ease score was changed from 0.1 to 1 to include more output. Ease score is a conservative adjustment to the Fisher exact probability. It weights significance in favor of the association supported by more genes. DAVID then generates functional annotation chart which lists annotation terms and their associated genes under study.

Result

Metformin reduced cell proliferation and increased G_1 arrest in colon cancer cell lines

Recently, there have been some reports that metformin inhibits cell proliferation and induces cell cycle arrest [69]. Based on these studies, I investigated if metformin affects cell proliferation and the cell cycle in parental-(SNU-C5) or 5-Fu resistant colorectal cancer cell lines (SNU-C5_5FuR). To confirm the metformin effects on cell proliferation, I tested 3 different conditions: 5-Fu serial dilution treatment from 10 μg/mL, metformin serial dilution treatment from 100 mM, and combination treatment of 5-Fu serial dilution with 10 mM metformin. As shown in Figure 1, SNU-C5 was sensitive to 5-Fu since it was treated

with a 5-Fu serial dilution. However, SNU-C5_5FuR was merely changed to 5-Fu, which indicated that the drugs react to parental and 5-Fu resistant cell lines. Both cell lines were affected by metformin, especially the 5-Fu resistant cell line. I confirmed the synergistic effect of combination treatment that leads to the serial dilution of 5-Fu and 10 mM metformin. The proliferation rate of SNU-C5 and SNU-C5_5FuR cell lines was effectively reduced by the combination treatment of 5-Fu and metformin. Consequently, all combination doses had the synergistic effect (Figure 1C, combination index (CI) <1). At the lowest 5-Fu dose and 10 mM metformin combination, the proliferation rate decreased 57.5% in SNU-C5 and 60.2% in SNU-C5_5FuR, compared to the 5-Fu only treatment. At the highest 5-Fu dose and 10 mM metformin combination, the proliferation rate decreased 17% and 57% in SNU-C5 and SNU-C5_5FuR, respectively. In this study, I suggested that metformin reduced cell proliferation, increased sensitivity to 5-Fu in SNU-C5_5FuR.

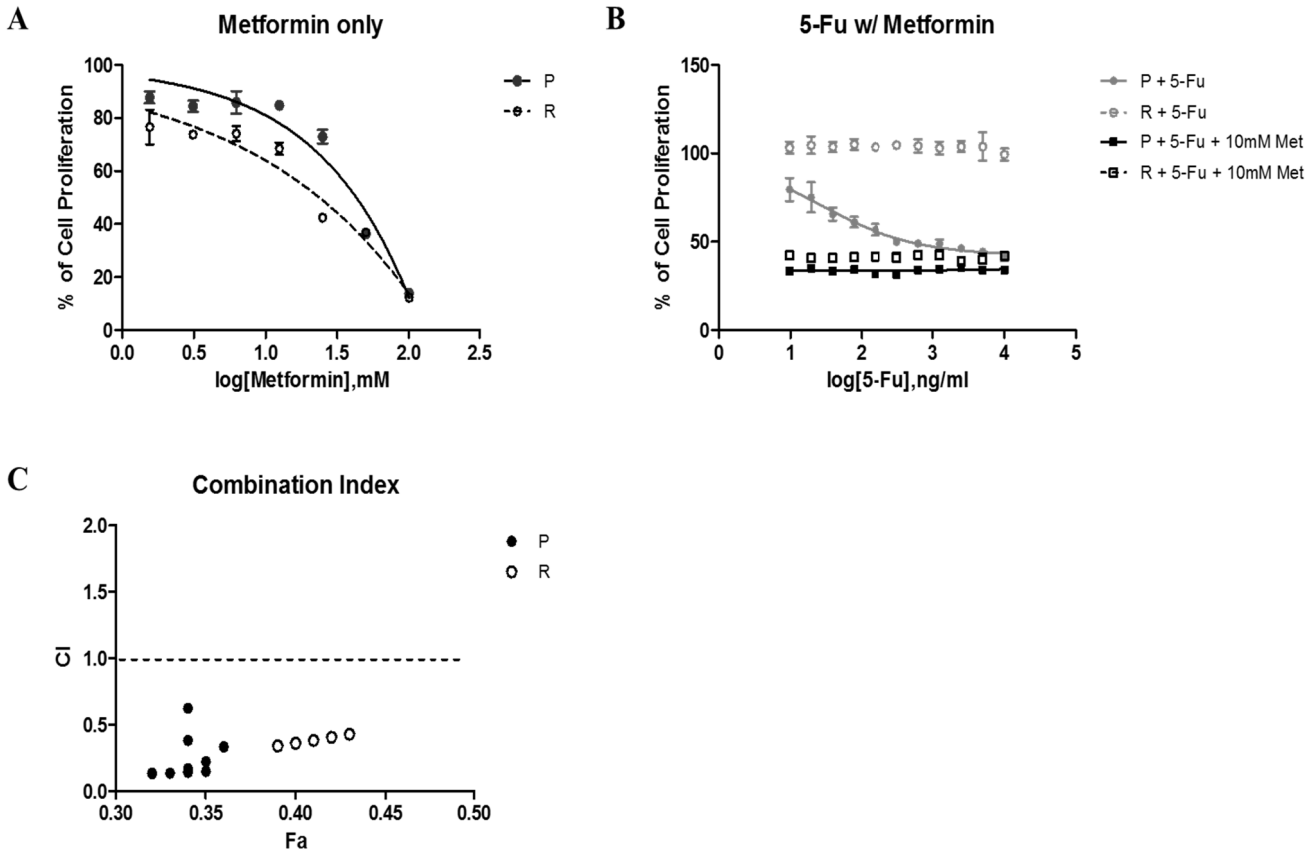

A
Metformin only
% of Cell Proliferation
log[Metformin],mM
P
R
B
5-Fu w/ Metformin
% of Cell Proliferation
log[5-Fu],ng/ml
P + 5-Fu
R + 5-Fu
P + 5-Fu + 10mM Met
R + 5-Fu + 10mM Met
C
Combination Index
CI
Fa
P
R

Figure 1. The relative cell proliferation rate as treated with 5-Fu, metformin, and combination 5-Fu with metformin.

Metformin only treated with serial dilution from 100 mM (A), 5-Fu only or both 5-Fu and 10 mM Metformin (B). 5-Fu was treated with serial dilution from 10 μg/mL. The cell proliferation rate was confirmed after a 72 h treatment of drugs using Ez-Cytox, which assessed NADH-dehydrogenase in live cells. (C) represents the combination index (CI) that is calculated using Compusyn (http://www.combosyn.com/). CI<1 means the synergistic effect of combination 5-Fu and metformin treatment, CI=1 is an additive effect, and CI>1 is the antagonistic effect. The data are represented as mean ± SD three independent experiments.

P; parental cell line, SNU-C5, R; resistant cell line, SNU-C5_5FuR, 5-Fu; serial dilution of 5-Fu, Met; Metformin

Next, I confirmed apoptotic proteins whether metformin leads to cell death or cell proliferation. By treatment with 50 mM of metformin, the cleaved caspase-3 and PARP were increased in a similar manner in both cell lines, SNU-C5 and SNU-C5_5FuR, when compared to lower dose (10 mM) of metformin (Figure 2A). Therefore, I speculated that metformin induced cell death. In SNU-C5, the 5-Fu treatment and combination 5-Fu and metformin treatment, cleaved caspase-3 and PARP, respectively, were significantly increased. In SNU-C5_5FuR, however, the apoptotic proteins were induced by metformin or 5-Fu and metformin combination treatment. I also substantiated these results by analyzing Annexin V positive cells. Thus, metformin decreased cell proliferation and increased cell death with induced apoptotic proteins. It also has higher synergistic effects with 5-Fu in SNU-C5_5FuR than SNU-C5. To confirm if metformin affects the cell cycle, I analyzed the 1 μg/mL of 5-Fu or 50 mM of metformin treatment and 5-Fu and metformin combination treatment to SNU-C5 and SNU-C5_5FuR (Figure 3). The G_0/G_1 percentages in both SNU-C5 and SNU-C5_5FuR cell lines were increased to 21.25% and 30.07%, respectively, when treated with metformin. As the combination treatment to SNU-C5_5FuR,

27.23% of the cells were arrested at the G_0/G_1 phase, which means that metformin occurred during G_0/G_1 arrest. These results suggested that both 5-Fu and metformin influenced SNU-C5 in cell proliferation and death. In contrast, only metformin or combination 5-Fu and metformin treatment influenced SNU-C5_5FuR. Consequently, I speculated that metformin could have synergistic effects on 5-Fu.

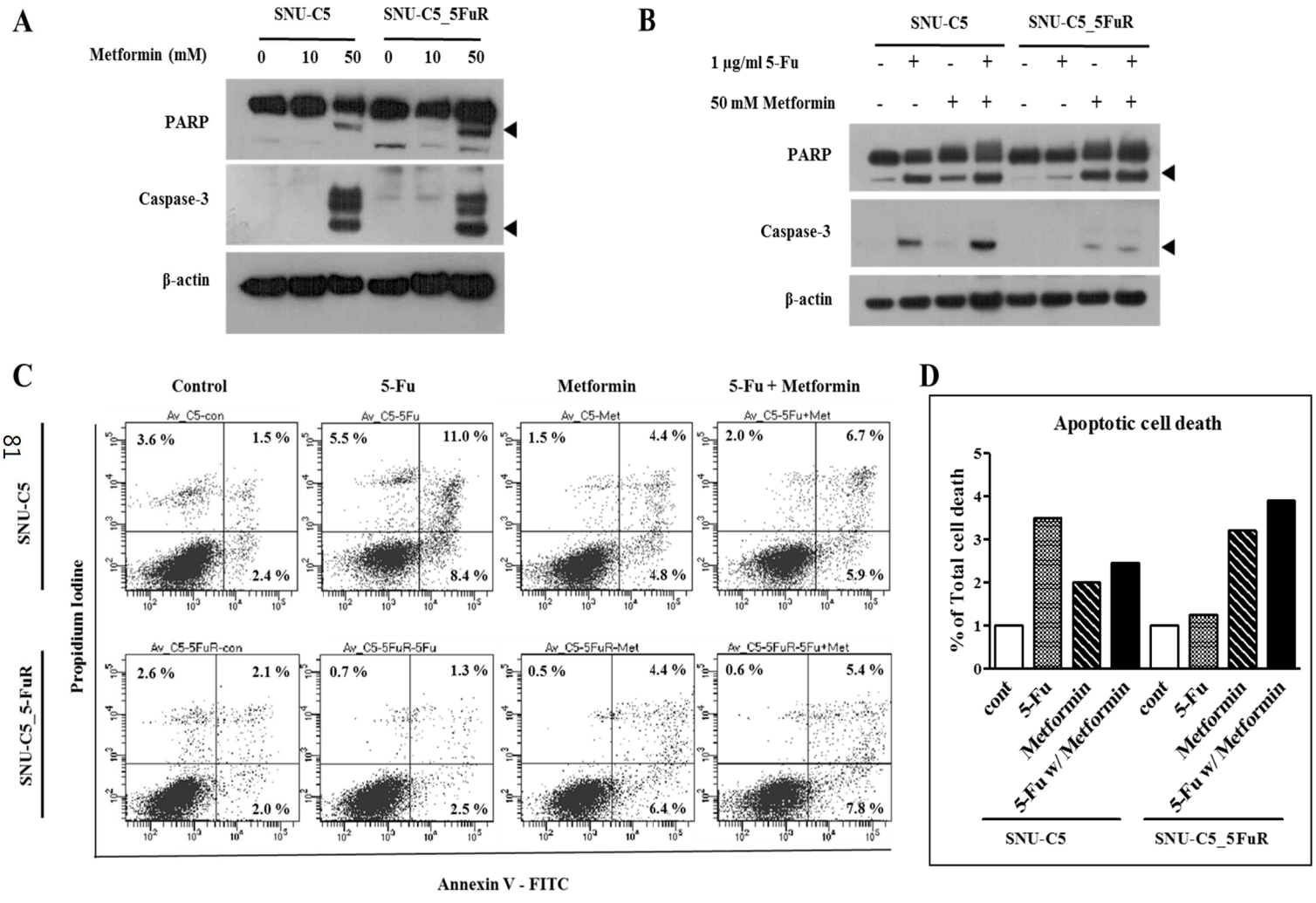
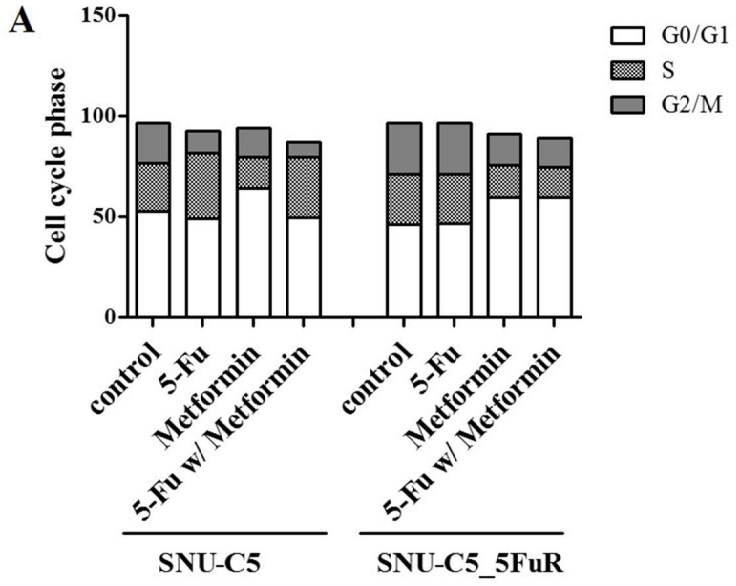

A
SNU-C5
SNU-C5_5FuR
Metformin (mM)
0 10 50 0 10 50
PARP
Caspase-3
β-actin
B
SNU-C5
SNU-C5_5FuR
1 μg/ml 5-Fu
50 mM Metformin
PARP
Caspase-3
β-actin
C
Control
5-Fu
Metformin
5-Fu + Metformin
SNU-C5
SNU-C5_5-FuR
Propidium Iodine
Annexin V - FITC
Av_C5-con
3.6 %
1.5 %
2.4 %
Av_C5-5Fu
5.5 %
11.0 %
8.4 %
Av_C5-Met
1.5 %
4.4 %
4.8 %
Av_C5-5Fu+Met
2.0 %
6.7 %
5.9 %
Av_C5-5FuR-con
2.6 %
2.1 %
2.0 %
Av_C5-5FuR-5Fu
0.7 %
1.3 %
2.5 %
Av_C5-5FuR-Met
0.5 %
4.4 %
6.4 %
Av_C5-5FuR-5Fu+Met
0.6 %
5.4 %
7.8 %
D
Apoptotic cell death
% of Total cell death
cont
5-Fu
Metformin
5-Fu w/ Metformin
cont
5-Fu
Metformin
5-Fu w/ Metformin
SNU-C5
SNU-C5_5FuR

Figure 2. Apoptotic proteins and Annexin V assay as treated with 5-Fu, metformin, and combination treatment.

The expression levels of apoptotic pathway proteins as treated with various doses of metformin (A) and combination 1 μg/mL of 5-Fu and 50 mM of metformin treatment (B) by western blotting. The arrow head indicates the active form of PARP or caspase-3. Apoptotic cell death detected by Annexin V/PE staining as treated with 0.5 μg/mL of 5-Fu and 10 mM of metformin (C). D represents the percentage of Annexin V-positive population.


A
150
100
50
0
Cell cycle phase
G0/G1
S
G2/M
control
5-Fu
Metformin
5-Fu w/ Metformin
control
5-Fu
Metformin
5-Fu w/ Metformin
SNU-C5
SNU-C5_5FuR

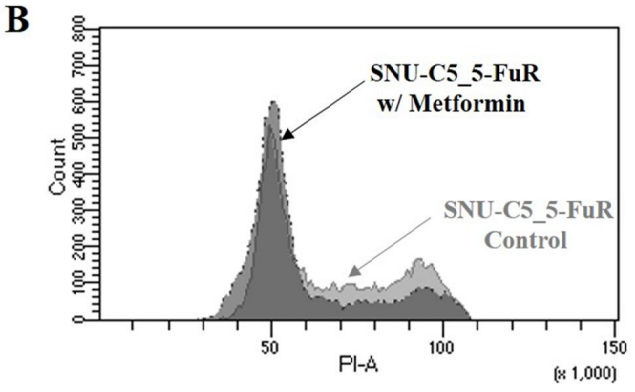

B
SNU-C5_5-FuR
w/ Metformin
SNU-C5_5-FuR
Control
Count
PI-A
(x 1,000)

Figure 3. Cell cycle analysis of SNU-C5 and SNU-C5_5FuR.

When treated with 1 μg/mL of 5-Fu and 50 mM of metformin as well as combination 5-Fu and metformin. The bar graphs indicate the changes in the cell cycle progression (A) and raw data of cell cycle distribution in SNU-C5_5FuR cell lines (B).

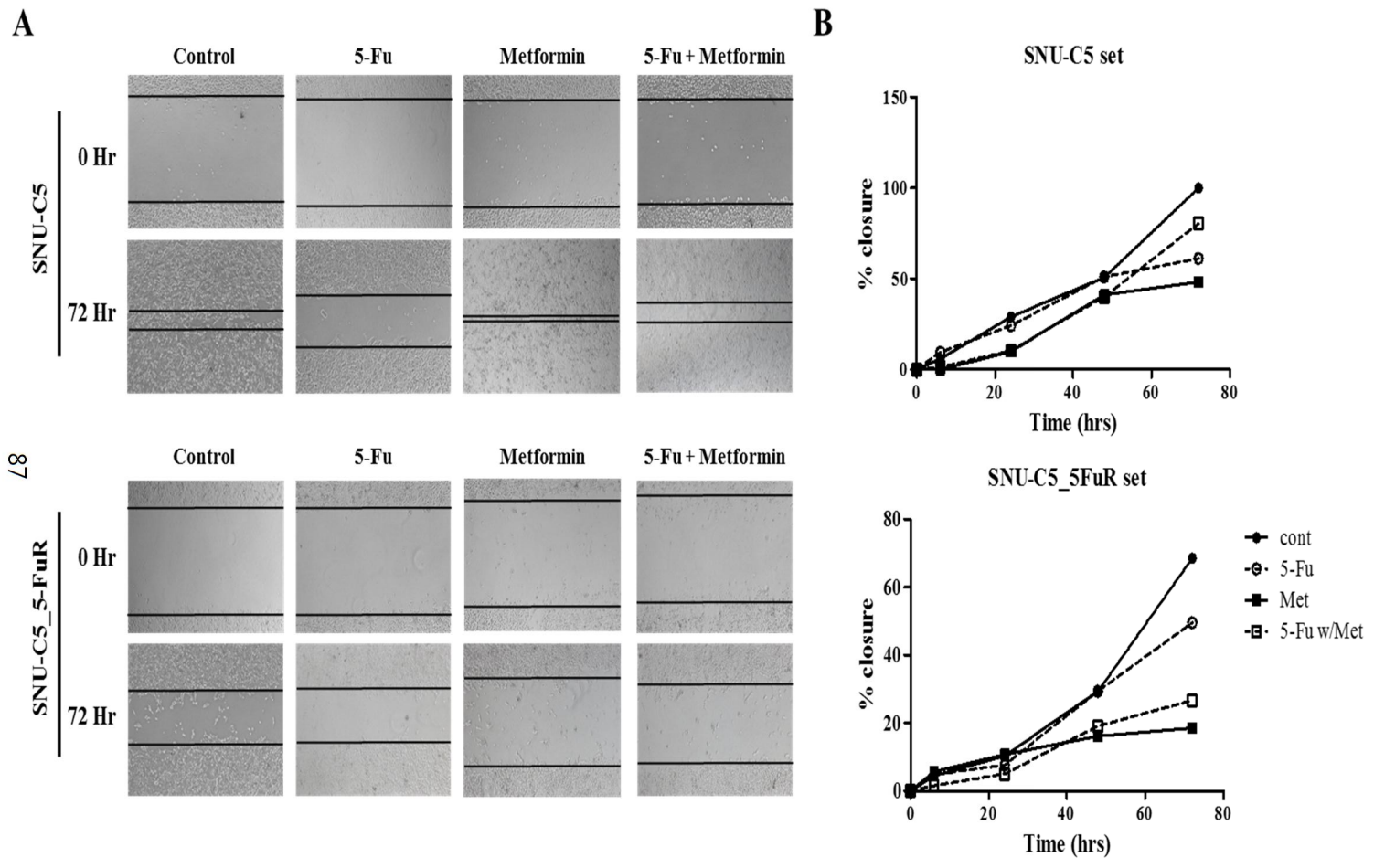
Metformin influenced cell migration, clonogenicity and angiogenesis

To investigate the metformin effects on cell migration and clonogenic ability, I performed wound healing and clonogenic assays. 0.5 μg/mL of 5-Fu and 10 mM of metformin, and the combination treatment of 5-Fu and metformin were treated to SNU-C5 and SNU-C5-5FuR cell lines, respectively. After 0, 6, 24, 48, and 72 h, I confirmed the relative cell migration rate. As shown in Figure 4A and B, both 5-Fu and metformin influenced the cell migration rate. Compare to SNU-C5 control, the migration rate decreased at 38.78% and 51.65% when treated with 5-Fu and metformin, respectively. It was also decreased 19.51% due to the combination treatment of 5-Fu and metformin in SNU-C5 parental cell line. For SNU-C5_5FuR, the migration rate decreased 27.78%, 72.95%, and 61.04% when treated with 5-Fu, metformin, and combination, respectively. SNU-C5_5FuR cell line tended to slow migration when compared with SNU-C5. The two cell lines had different cell migration rates when treated with drugs. SNU-C5 was influenced by 5-Fu more than metformin, while SNU-C5_5FuR was more sensitive to metformin. The cell migration capacity has influenced metformin more than 5-Fu in this cell line. The data showed that metformin might influence cell

migration and that combination treatment with 5-Fu was effective in targeting 5-Fu resistant cancer cell line. Metformin also inhibits metastatic behavior like angiogenesis in many cancers [79, 80].

The clonogenic ability was comparable with cell migration patterns when treated with drugs: SNU-C5 was affected by 5-Fu more than metformin. Metformin treatment and combination 5-Fu and metformin effectively reduced clonogenic ability in SNU-C5_5FuR cell lines (Figure 4C, D).

To investigate metformin on angiogenesis, I also confirmed HIF-1α and VEGF. I found that HIF-1α expression decreased when treated with 5-Fu in SNU-C5 and with metformin in SNU-C5_5FuR. As a result, I suggested SNU-C5_5FuR as more sensitive to metformin than SNU-C5. Additionally, metformin affected cell migration ability and expression of angiogenesis related proteins.


A
Control
5-Fu
Metformin
5-Fu + Metformin
SNU-C5
0 Hr
72 Hr
Control
5-Fu
Metformin
5-Fu + Metformin
SNU-C5_5-FuR
0 Hr
72 Hr
B
SNU-C5 set
% closure
Time (hrs)
SNU-C5_5FuR set
% closure
Time (hrs)
cont
5-Fu
Met
5-Fu w/Met

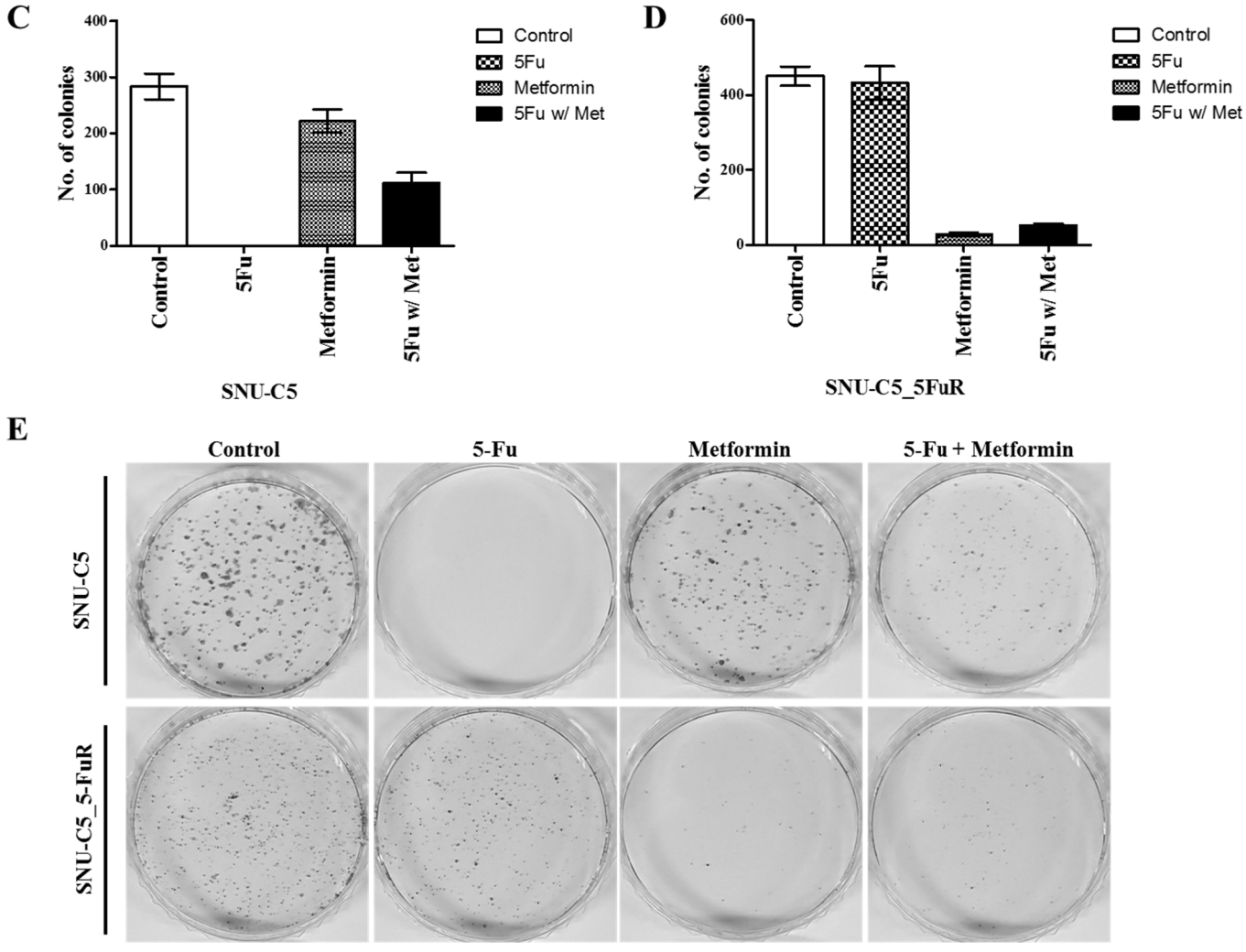

C
No. of colonies
400
300
200
100
0
Control
5Fu
Metformin
5Fu w/ Met
SNU-C5
D
No. of colonies
600
400
200
0
Control
5Fu
Metformin
5Fu w/ Met
SNU-C5_5FuR
E
Control
5-Fu
Metformin
5-Fu + Metformin
SNU-C5
SNU-C5_5-FuR

Figure 4. Metformin affected wound healing capacity and clonogenicity.

The wound healing assay and clonogenic assay were performed by 0.5 μg/mL of 5-Fu and 10 mM of metformin as well as combination 5-Fu and metformin treatment. For the migration assay, 50,000 cells/well were seeded, wounded, and then treated with PBS (as control), 5-Fu, and metformin. The wound was observed at 0, 6, 24, 48, and 72 h. A represents the taken phase-contrast picture images at 0 and 48 h. B shows the calculated cell migration where the black closed circle is control, open circle is 5-Fu treatment, closed square is metformin, and open square is combination treatment. For clonogenic assay, 0.5×10^3 cells are pre-treated by 5-Fu w/ or w/o metformin and seeded in a 60 mm dish. After 14 days, the colonies are counted by staining with crystal violet. The experiments are performed three times. C and D represent the number of SNU-C5 and SNU-C5_5FuR coloines, respectively. The data are represented as mean ± SD three independent experiments. E shows the picture images of those colonies.

Metformin's effect on AMPK/mTOR axis and NF-κB pathway

The well-known metformin mechanism was via the AMPK/mTOR axis that inhibits cellular metabolism and protein synthesis by metformin [71]. Metformin activates the AMPK pathway, which inhibits mTOR. In addition, the NF-κB pathway is known to affect metformin [81]. To confirm the metformin action pathway, detection of protein levels by western blot analysis was performed. As shown in Figure 5, phospho-AMPKα increased and phospho-mTOR decreased when treated with metformin, especially in SNU-C5_5FuR cell line. In contrast, no phospho-AMPKα augmentation was detected in SNU-C5 cell line. The NF-κB pathway decreased when treated with a combination of the 5-Fu and metformin in both cell lines as opposed to a single treatment of 5-Fu. In this study, metformin inhibits cell proliferation and migration via the AMPK/mTOR axis and NF-κB pathway was confirmed. In addition, the SNU-C5_5FuR cell line is more sensitive to metformin than SNU-C5.

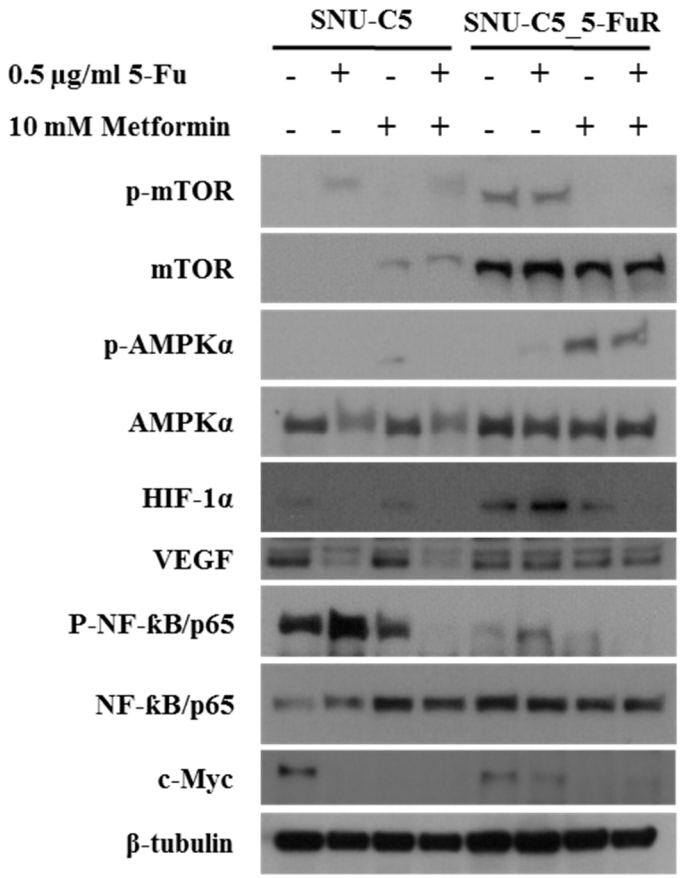
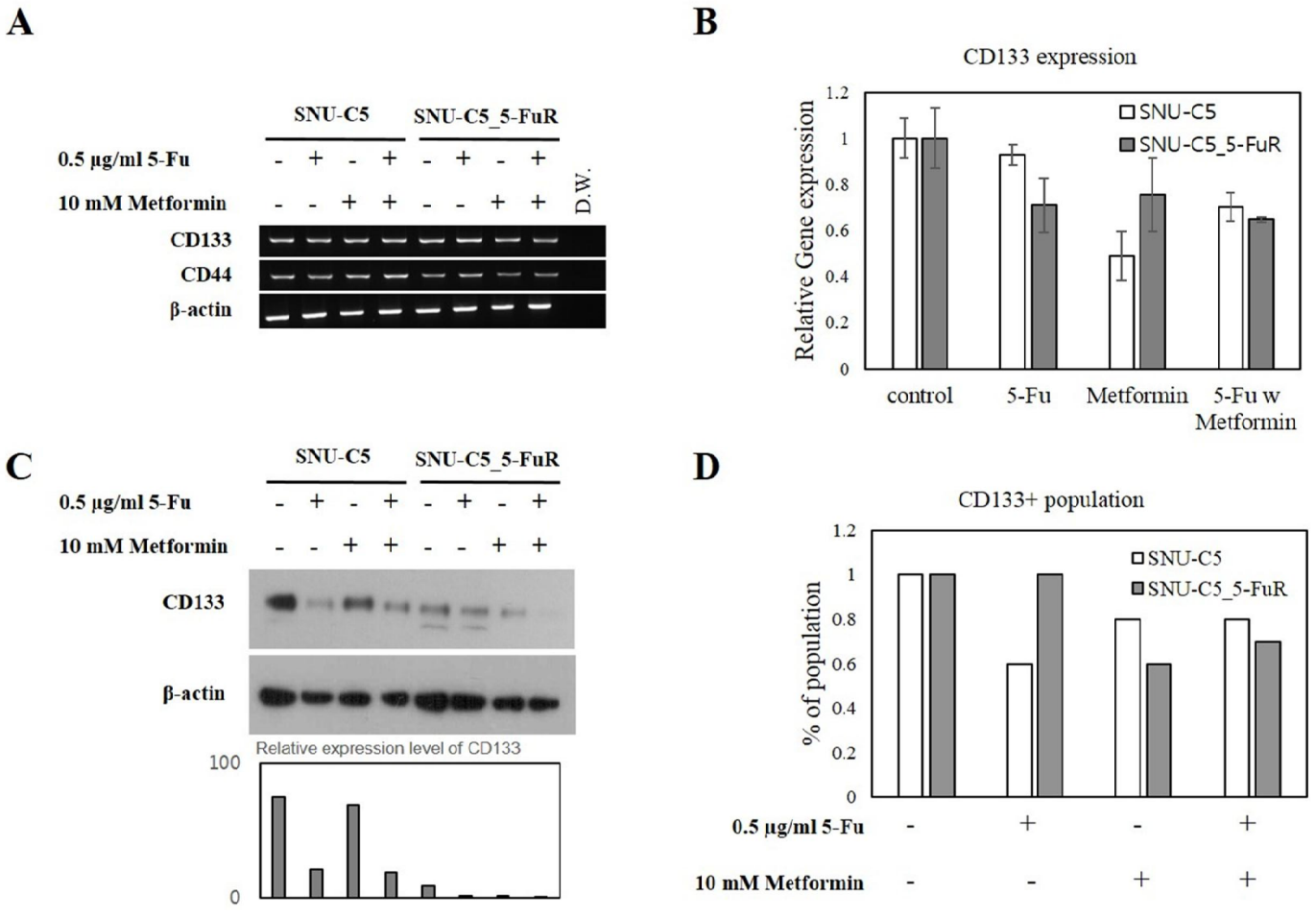


Figure 5. Metformin effects on AMPKα/mTOR axis pathway, and NF-ĸB pathway effector proteins.

AMPK/mTOR signaling pathway is well-known mechanism of metformin. Total AMPK and mTOR, and their phosphorylation form were determined by western blot analysis when treated with 5-Fu and/or metformin. Also, NF-ĸB and angiogenesis related related proteins, HIF-1α, VEGF were confirmed. β-tubulin was used an a internal control.

Metformin influenced cancer stem cell population and tumor sphere formation

Recently, rising evidences suggests that cancer stem cells (CSCs) are related to drug resistance in cancers [82, 83]. Based on these theory, I investigated the changes in cancer stem cell population using CD133 and CD44, which are well known colorectal cancer stem cell surface markers caused by 5-Fu, metformin, and combination treatments. In SNU-C5 and SNU-C5_5FuR, 5-Fu and metformin reduced the transcriptional and translational expression levels of CD133 (Figure 6A-C). In SNU-C5, 71.8%, 15.0%, and 75.3% of the CD133 protein expression decreased when treated with 5-Fu, metformin, and combination, respectively. In SNU-C5_5FuR, 87.9%, 90.1%, and 93.4% decreased when treated with 5-Fu, metformin, and combination, respectively. I performed fluorescence-activated cell sorter (FACS) analysis to verify the above data. As shown in Figure 6D, SNU-C5 was influenced by 5-Fu and SNU-C5-5FuR more than metformin. I suggest that metformin influences cancer stem cell populations according to these results.


A
SNU-C5
SNU-C5_5-FuR
0.5 μg/ml 5-Fu
10 mM Metformin
D.W.
CD133
CD44
β-actin
B
CD133 expression
Relative Gene expression
SNU-C5
SNU-C5_5-FuR
control
5-Fu
Metformin
5-Fu w Metformin
C
SNU-C5
SNU-C5_5-FuR
0.5 μg/ml 5-Fu
10 mM Metformin
CD133
β-actin
Relative expression level of CD133
D
CD133+ population
% of population
SNU-C5
SNU-C5_5-FuR
0.5 μg/ml 5-Fu
10 mM Metformin

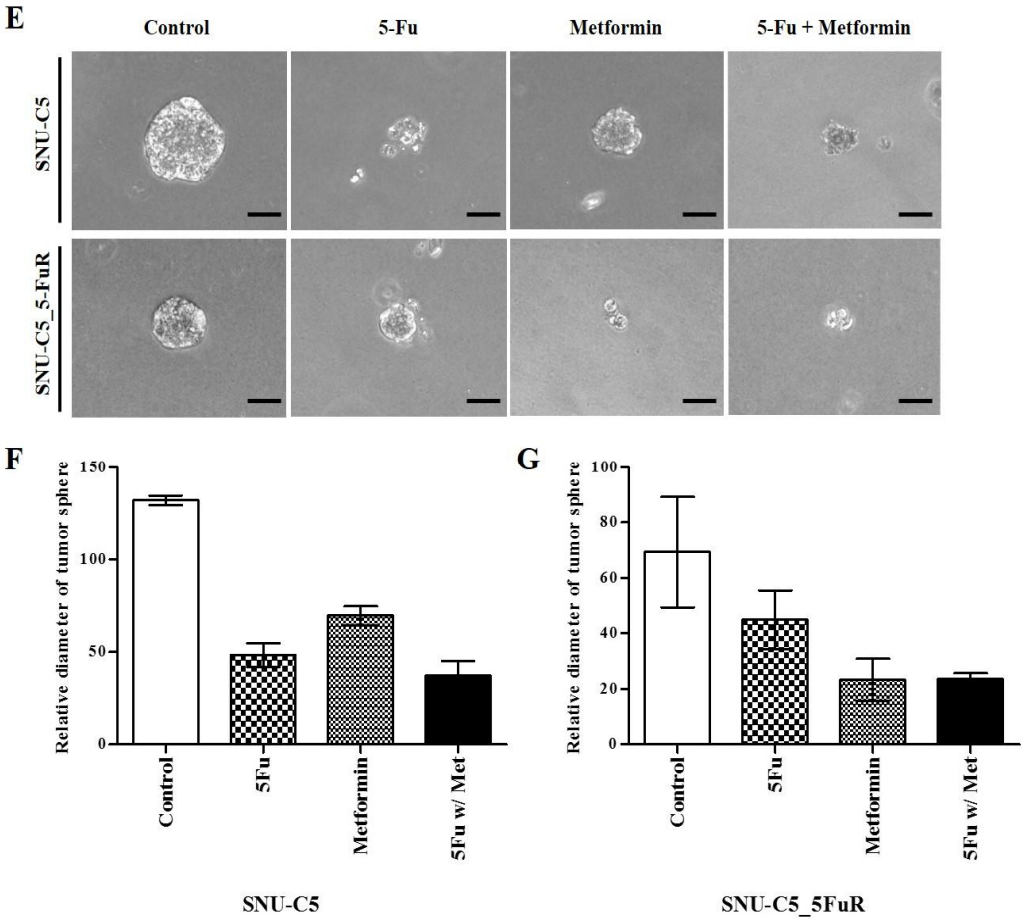

E
Control
5-Fu
Metformin
5-Fu + Metformin
SNU-C5
SNU-C5_5-FuR
F
Relative diameter of tumor sphere
150
100
50
0
Control
5Fu
Metformin
5Fu w/ Met
SNU-C5
G
Relative diameter of tumor sphere
100
80
60
40
20
0
Control
5Fu
Metformin
5Fu w/ Met
SNU-C5_5FuR

Figure 6. Metformin effects on cancer stem cell marker (CD133) expression level and tumor sphere formation.

With 0.5 μg/mL of 5-Fu and 10 mM of metformin by RT-PCR (A), quantitative-RT-PCR (B), western blot analysis (C), and FACs analysis (D). Quantitative RT-PCR data was normalized by β-actin expression level and then calculated ddCt value using 7300 System SDS v1.4. Software. Western blot band intensities were calculated by ImageJ (the graph) in the lower bar graph. After 3D culture for tumor sphere formation with RGF-BME, microscopical analysis (magnification × 250, Scale Bar=50μm) (E). F and G represent relative diameter of tumor sphere measured using Image J.

Metformin reduced DNA replication machinery genes in 5-Fu resistant cancer cell line

To investigate the other mechanisms of metformin action on 5-Fu resistant cancer cell line, I performed RNA sequencing. After conducting RNA-seq, I sorted genes that have more than two fold changes and have p-values less than 0.05, as determined in accordance with the absolute value of RPKM and reads quality. A total of 658 genes from 25,269 genes were sorted. By using iVariantGuide software, pathways affected by metformin treatment in RNA levels were detected. As shown in Figure 7C and D, DNA helicase activity was mostly affected while DNA replication machinery and cell cycle regulation genes were significantly reduced. I also verified protein expression levels related to DNA replication machinery: MCM2 and PCNA (Figure 7E). Interestingly, MCM2 and PCNA were reduced more when SNU-C5_5FuR was treated with metformin rather than SNU-C5. Chk1 and Chk2 are essential proteins for DNA damage response and cell cycle check point, which were reduced in metformin treated parental-, and resistant cancer cell lines. Phospho-Chk1 was reduced when treated with metformin, however, phospho-Chk2 merely changed in both cell lines. In summary,

metformin selectively affected the DNA damage response and DNA replication, especially in 5-Fu resistant colon cancer cell line.

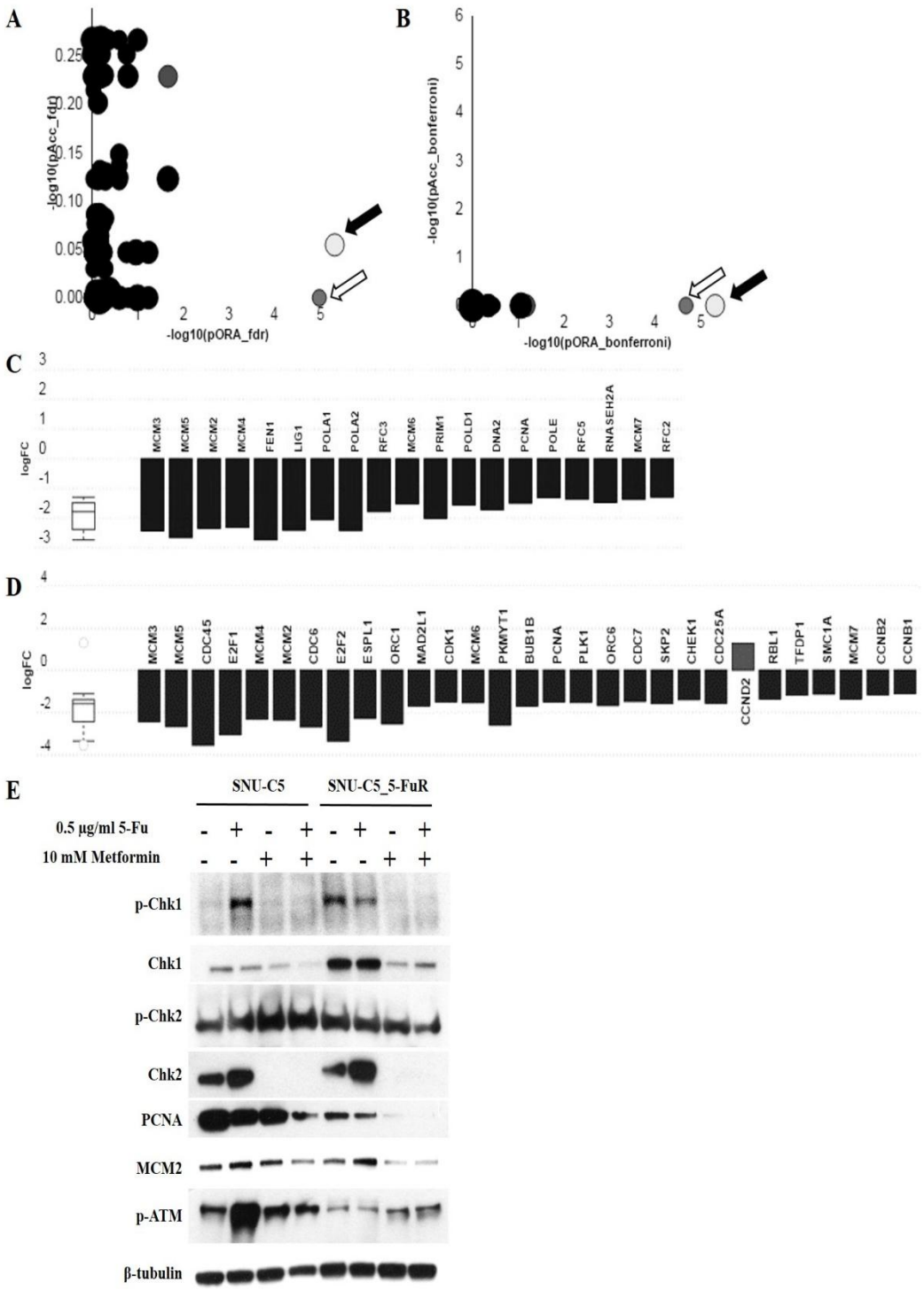

A
-log10(pAcc_fdr)
-log10(pORA_fdr)
B
-log10(pAcc_bonferroni)
-log10(pORA_bonferroni)
C
logFC
MCM3 MCM5 MCM2 MCM4 FEN1 LIG1 POLA1 POLA2 RFC3 MCM6 PRIM1 POLD1 DNA2 PCNA POLE RFC5 RNASEH2A MCM7 RFC2
D
logFC
MCM3 MCM5 CDC45 E2F1 MCM4 MCM2 CDC6 E2F2 ESPL1 ORC1 MAD2L1 CDK1 MCM6 PKMYT1 BUB1B PCNA PLK1 ORC6 CDC7 SKP2 CHEK1 CDC25A CCND2 RBL1 TFDP1 SMC1A MCM7 CCNB2 CCNB1
E
SNU-C5
SNU-C5_5-FuR
0.5 μg/ml 5-Fu
10 mM Metformin
p-Chk1
Chk1
p-Chk2
Chk2
PCNA
MCM2
p-ATM
β-tubulin

Figure 7. Metformin influenced to DNA replication machinery genes.

As a result of RNA seq., DNA replication (KEGG: 03030) (black arrows in A and B), and mitotic cell cycle (KEGG: 04110) (white arrows in A and B) genes were downregulated when treated with metformin. A is the gene correction by False Discovery Rate (FDR) and B is by Bonferroni correction.

All the genes from DNA replication pathway (B) and mitotic cell cycle pathway (C) were represented in terms of their measured fold change (y-axis) and accumulation (x-axis). The related protein level was detected by western blot analysis (D).

Discussion

The key research question of this study was to determine if metformin has anticancer effects and if it acts as a chemosensitizer for recovering chemo-resistance in colorectal cancer line, SNU-C5, and 5-Fu resistant cell line.

I first performed cell proliferation assays and cell cycle analysis after treating 5-Fu or metformin and combination 5-Fu and metformin treatment to SNU-C5 and SNU-C5_5FuR. As shown in Figure 1, the SNU-C5 cell line was more sensitive than SNU-C5_5FuR at different 5-Fu concentrations. When treated with different 5-Fu doses, the proliferation rate of the resistant cell line did not significantly change. On the contrary, the parental cell line decreased. At 10 μg/mL of 5-Fu, the highest dose, cell proliferation decreased about 39% in SNU-C5. Both cell lines, SNU-C5, and SNU-C5_5FuR, decreased cell proliferation affecting metformin in treatment (Figure 1A). Thus, I wonder if metformin and 5-Fu has synergistic effects. For the combination treatment, serial dilution from 10 μg/mL of 5-Fu and 100 mM metformin were treated to SNU-C5, and SNU-C5_5FuR. Combination treatment has synergistic effects on reducing cell proliferation at every

concentration of 5-Fu for both SNU-C5 and SNU-C5_5FuR, as confirmed by CI value calculation (<1) (Figure 1C). According to Chen Qu. *et al*, metformin has reversal effects on chemo-resistance in breast cancer cells [84]. Corresponds to these result, our data suggested that the 5-Fu resistant-cell line, SNU-C5_5FuR, was more sensitive with metformin than the parental cell line. The increased expression level of apoptotic proteins was validated these data. As shown in Figure 2B, the active form of PARP and caspase-3 expressed with cell death were mainly induced by 5-Fu in SNU-C5 and metformin in SNU-C5-5FuR.

This data showed that metformin reduces cell proliferation via increased sensitivity to 5-Fu and apoptotic protein expression, especially in 5-Fu resistant-colorectal cancer cell lines. I observed metformin affecting the cell cycle, migration rate, and clonogenicity as in the preceding data. In SNU-C5_5FuR cells, treatment with 5-Fu alone had less impact on cellular behavior like the cell cycle. In contrast, treatment with metformin and combination increased cell death, cell cycle arrest at G_0/G_1 phase, and decreased rate of migration and clonogenicity. The metformin effects had a discrepancy between parental cell line and 5-Fu resistant cell line. SNU-C5 was less sensitive to metformin than 5-Fu.

With these results, I suggested the selective response of metformin to 5-Fu resistant cell lines. In addition, metformin has synergistic effects with 5-Fu (Figure 4). The VEGF expression level was enhanced by of the cooperation between c-Myc and HIF-1α [85], while the expression of c-Myc was regulated by microRNA-33a from metformin treatment [86]. HIF-1α is a key regulator of cancer metabolism particularly in anaerobic condition. In addition, both of HIF-1α and VEGF are involved in angiogenesis, which is an important feature of cancer metastasis for cellular behavior. Thus, I confirmed HIF-1α, and VEGF to find that these proteins are regulated by metformin. As a result, I suggested that metformin influence cancer metastatic behavior. To date, there has been clinical trials of metformin for chemoprevention with angiogenic effect in Barretts' s metaplasia, colorectal adenoma, and prostate cancer [79]. In addition to this data, metformin has been suggested to have anti-angiogenic effects. Furthermore, there is evidences that metformin regulates the cell cycle by G_0/G_1 arrest and cell death via AMPK pathway. When treated with metformin, phospho-AMPK increased in SNU-C5_5FuR cell while phospho-mTOR was decreased (Figure 5). This confirmed that the activated AMPK signaling

pathway might play a key role in the 5-Fu resistant cell line. Metformin suppresses MDR1 expression in the concomitant inhibiting nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) [81, 87, 88]. In this data shows that metformin inhibited NF-κB pathway and the combination treatment of 5-Fu and metformin has significantly affected the activation. However, used cell lines SNU-C5 and SNU-C5_5FuR did not expressed MDR1 (data are not shown).

Besides the AMPK-mTOR axis and NF-κB pathway, I wondered if the other metformin pathways acted on 5-Fu resistant cancer cells. Therefore, I confirmed the cancer stem cell population from the evidences that metformin inhibits cancer cell proliferation via targeting cancer stem cell population [72, 89, 90]. CD133, also known as prominin-1 or AC133, is a 7-transmembrane surface maker of cancer stem cells, especially in colon cancer [91]. I observed that metformin affects the clonogenicity capacity, as shown in Fig. 4E. This may demonstrated that metformin is related to the self-renewal ability with the stemness characteristic The CD133 positive cell population and expression level of CD133 was reduced in SNU-C5 and SNU-C5_5FuR, and these were related with tumor sphere formation (Fig. 6). Notably,

CD133 expression and ability of tumor sphere formation in SNU-C5_5FuR was significantly reduced by metformin and combination treatment of 5-Fu and metformin.

Additionally, I performed RNA sequencing using SNU-C5_5FuR control and metformin treated samples to confirm the metformin regulated pathway in the 5-Fu resistant cell line. As a result, I confirmed DNA polymerization complex genes, such as MCM2, PCNA, and cell cycle related genes, that were depleted by metformin treatment in RNA and protein level in 5-Fu resistant cancer cell line. The MCM complex is a major component in eukaryotic DNA replication machinery, which interacts with Chk1 as DNA becomes damaged [92]. Consequently, metformin was reported to damage DNA, activated the intracellular ATM/Chk2 checkpoint, and regulation the cell cycle [93]. MCM2, PCNA, and Chk1 were down regulated and ATM was activated by metformin in the SNU-C5_5FuR cell line. Therefore, the previous cell cycle arrest data increased 5-Fu sensitivity and induced metformin in 5-Fu resistant cell line due to DNA damage. Interestingly, there was less influence on the MCM2 and PCNA expression level due to metformin treatment on the SNU-C5 parental cell line, which means that metformin selectively

affects DNA replication. This is particularly present in the SNU-C5_5FuR cell line.

In conclusion, metformin has shown synergistic effects in combination with the conventional chemotherapeutic agent, 5-Fu through DNA damage and inhibition of DNA replication machinery, particularly in 5-Fu resistant cancer cell line. Therefore, I speculate that metformin could be used in adjuvant chemotherapeutics without severe side effects.

GENERAL DISCUSSION AND CONCLUSION

In this study, I investigated CEACAM1 expression level as treated with 5-Fu, and metformin effects in gastrointestinal cancer cell lines, and their 5-Fu resistant cell lines

Between SNU-638, SNU-C5, and their 5-Fu resistant cancer cell lines has different response to 5-Fu treatment. First, 5-Fu resistant cell lines were merely affected to cell proliferation and cell cycle distribution than parental cell lines. Second, there were varies gene expression level, e.g. CEACAM1, which known as tumor suppressor gene, and apoptotic proteins such as PARP and caspase-3. Due to CEACAM1 was increased as dose-, and time dependent manner of 5-Fu in parental cell lines, I was speculated that CEACAM1 might be correlated with 5-Fu sensitivity. However, not only treatment with 5-Fu but also the other chemo-therapeutic agents, oxaliplatin, irinotecan, and radiation, were affected to CEACAM1 expression level. Through this, I supposed to CEACAM1 as a cell death related protein, especially anticancer drugs.

Thus I suggested that CEACAM1 might be applicable to prediction the sensitivity of chemotherapeutic agents.

Next, I was investigated how recover the 5-Fu resistance. Metformin, one of the type II diabetics target drug, was reported that has anticancer effect. I expected that metformin has additive or synergistic effect with 5-Fu. As treated with metformin or combination with 5-Fu and metformin, were influenced to reduction of cell proliferation, clonogenecity, cancer stem cell population, and induced cell cycle arrest, especially in 5-Fu resistant cancer cell line. Decrease of DNA replication machinery genes and mitotic cell cycle arrest genes were concomitant to metformin treatment. Due to these effects, I proposed that use of metformin as adjuvant of 5-Fu that might be reduction of 5-Fu resistance, with less clinical severe adverse effects.

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국문초록

5-Fluorouracil 내성 위장관암 세포에서 CEACAM1 매개 세포사멸 및 Metformin 항암효과에 관한 연구

김 성 희

서울대학교 대학원

협동과정 종양생물학 전공

위장관암은 한국인에서 높은 발병율과 사망율을 보이고 있다. 위장관암은 항암제 투여 및 방사선 조사가 수반되는 외과적 수술을 통하여 치료가 이루어진다. 항암제 치료 시, 일반적으로 5-Fluorouracil (5-Fu), oxaliplatin, irinotecan 등을 조합하여 환자에게 처방한다. oxaliplatin, irinotecan을 5-

Fu와 조합하여 처리하는 경우, 그 치료 효과는 5-Fu를 단독으로 처리하는 경우에 비하여 40-50%까지 증가하는 것으로 보고 되어있다. 그러나 항암제 내성을 원인으로 한 타 조직으로의 전이 및 재발이 빈번하게 나타나므로 이를 극복할 치료전략이 필요하다.

본 논문은 위장관암 세포주와 이 세포주의 5-Fu 내성세포주에서 5-Fu 처리에 따른 감수성 및 유전자 변화를 확인하고자 하였다. 그리고 5-Fu 내성을 극복할 수 있는 항암치료 보조제를 제안하고자 한다.

암 억제 유전자로 알려져 있는 Carcinoembryonic antigen-related cell adhesion molecule1 (CEACAM1) 의 경우, 5-Fu 처리 농도 및 시간에 의하여 모세포주에서만 발현 양이 변화 되었다. 따라서 해당 유전자가 항암제 감수성과 연관이 있을 것으로 예상 하였다. 그러나 oxaliplatin, irinotecan, 방사선 조사에 의해서도 CEACAM1 발현 양에 변화가 생겼으므로, 이것이 5-Fu에 의한 반응보다는 항암제 처리시 세포사멸 과정에서 나타나는 표현형이라고 생각 된다. 이를 통하여 5-Fu 등의 항암제 처리 시 변화되는 CEACAM1 발현양은 향후 세포사멸을 예측해 볼 수 있는 지표로서 활용이 가능할 것으로 판단된다.

대표적인 제 2형 당뇨병 치료제인 메트포르민은 최근 항암 효과가 있는 것으로 보고 되어있다. 메트포르민 역시 위장관암 세포주에서 CEACAM1 발현양을 증가 시켰으며, 특히 5-Fu와 함께 처리 한 경우, 5-Fu 내성세포주에서 세포증식 억제, 세포주기 정지 및 세포사멸을 증가시키는 등 항암제의 상승효과를 나타냈다. 이러한 작용은 메트포르민이 DNA 복제 및 세포주기 관련 유전자들을 감소시켰기 때문으로 확인 하였다. 일련의 결과를 토대로, 메트포르민이 임상적인 부작용을 최소화하여 5-Fu 내성을 감소시킬 수 있는 5-Fu의 보조치료제로 활용할 수 있음을 제안한다.

주요어: 위장관암, 5-Fluorouracil (5-Fu), oxaliplatin, irinotecan, 항암제 내성, Carcinoembryonic antigen-related cell adhesion molecule 1(CEACAM1), Metformin

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