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Evaluation of the antitumor effects of alternate-day 5-fluorouracil administration model using human gastric cancer cell lines

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Abstract

Alternate-day drug administration (ADDA) of S-1, of which the final antitumor product is 5-fluorouracil (5-FU), has been considered to be a good alternative to current standard continuous drug administration (CODA) because it reduces adverse effects that can occur. However, the antitumor efficacy of ADDA and its possible combinational regimens with other drugs have not been comprehensively studied. In the present study, a panel of gastric cancer cell lines were used to compare the antitumor effect of ADDA using 5-FU to CODA. The majority of cell lines demonstrated no difference in antitumor effects between ADDA and CODA in growth suppression and colony formation assays. Moreover, simultaneous administration of cis-diamminedichloroplatinum reduced the differences between ADDA and CODA in both assays. Although cell cycle changes in response to the drugs were regulated almost exclusively by drug type, rather than administration mode, the number of apoptotic and necrotic cells increased in both ADDA and CODA groups in 5-FU-sensitive cell lines. These results suggest that ADDA may be a reasonable treatment approach without compromising the antitumor effects of S-1; however, modulators for 5-FU should be carefully considered depending on the molecular profile of individual tumors.

Key words : *alternate-day drug administration; 5-FU, gastric cancer, growth suppression, colony formation, adjuvant chemotherapy*

Abbreviations

5-FU: five-fluorouracil; ADDA: alternate-day drug administration of 5-FU; CODA: continuous drug administration of 5-FU; CDDP: cis-diamminedichloroplatinum; RFS: relapse-free survival; GI_{50}: 50% growth inhibitory concentration; CI_{50}: 50% colony-formation inhibitory concentration; PPC: peak plasma concentration.

I. Introduction

One of the most recent prominent achievements in gastric cancer chemotherapy has been the therapeutic use of S-1 (TS-1, Taiho Pharmaceutical, Tokyo, Japan) [1, 2]. Curative gastrectomy followed by adjuvant

chemotherapy using S-1 as a single agent has demonstrated a significant improvement in both relapse-free survival and overall survival [2, 3]. However, the 5-year outcome analysis revealed that more than 30% of patients experience cancer relapse despite the therapy [2]. To amend the S-1 regimen, which is currently a continuous drug administration (CODA), it is important to consider both the antitumor effect and any adverse effects experienced by the patients who receive the chemotherapy. An alternate-day drug administration (ADDA) regimen seemed to show fewer adverse events than CODA without influencing the expected clinical outcomes [4]. However, the detailed biological mechanism on how an ADDA affects tumor growth remains to be elucidated.

The product of the antitumor activity of S-1 is 5-FU, and this activity is enhanced by gimeracil, which potently inhibits 5-FU degradation, and by oteracil, which reduces gastrointestinal toxicity by suppressing the activation of 5-FU in the alimentary tract [1]. As a consequence, the concentration of 5-FU produced from S-1 is maintained at a high level, thereby enhancing the antitumor activity and reducing adverse effects [5]. Therefore, the actual antitumor activity of S-1 at the cellular level is achieved through 5-FU. In fact, 5-FU has been used to examine the antitumor activity and toxicity of S-1 in *in vitro* assays [5, 6].

In the present study we evaluated the *in vitro* antitumor activity of 5-FU in an ADDA model using a panel of gastric cancer cell lines. In principle, we sought to determine whether the antitumor effect of 5-FU using ADDA is equivalent to CODA in terms of growth suppression and colony formation. We further evaluated the mechanism of growth by measuring cell cycle distribution, cell death induction, invasion/migration ability, and protein level alterations in response to the drug administration. Based on previous reports, we hypothesized that 5-FU should have sufficient antitumor activity when using ADDA against most cell lines [6], but that some 5-FU insensitive tumor lines may exist. For those tumors, pharmacological modulation of 5-FU may be necessary based on the mechanisms of insensitivity [5]. In fact, one of the most challenging tasks is to identify tumors that are insensitive to S-1. However, to date a very limited number of sensitivity prediction markers have been reported for S-1 or 5-FU [7]. In addition to the comparison of ADDA and CODA, we also investigated the effect of continuous CDDP administration as a modulator [5, 8]. The results of these investigations provide justification for ADDA as a potential alternative to CODA based on biological mechanisms and may provide evidence for the utility of CDDP as a modulator to S-1.

II. Materials and Methods

1. Gastric cancer cell lines and drugs

Nine human gastric cancer cell lines were used for the study (Table 1). GCIY, H111TC, KE39, Kato-III, MKN7, MKN45, MKN74, and NUGC-4 were obtained from the RIKEN BioResource Center Cell Bank (Tsukuba, Japan). IWT-1 was established in our laboratory, and the use of this cell line was approved by the institutional review board of Iwate Medical University (HG H25-15, [9]). All cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine

Table 1. Characteristics of the gastric cancer cell lines.

Cell name	Gender	Age at sampling	Doubling time in culture (hours) [references]	Origin
GCIY	Female	Not available	55 [34]	Primary
H111TC	Not available	Not available	72	Primary
KE39	Male	77	38.4 [35]	Lymph node metastasis
Kato III	Not available	Not available	36 [36]	Primary
IWT-1	Male	58	22.7	Peritoneal dissemination
MKN7	Male	39	35 [22]	Lymph node metastasis
MKN45	Female	62	22.9 [37]	Liver metastasis
MKN74	Male	37	17 [22]	Liver metastasis
NUGC-4	Not available	Not available	36.1 [38]	Primary

serum (FBS) in a 37℃ incubator with 5% CO_2. The drug solutions of 5-FU (Kyowa Kirin, Tokyo, Japan) and CDDP (Randa® inj, Nippon Kayaku, Tokyo, Japan) were purchased from commercial sources. The doubling time of cells was measured by seeding 1.0-2.0 × 10^4 cells/well of a 24-well plate and monitoring the cell number every 24 hours for a period of 96 hours with a Tali® Image-based Cytometer (Life Technologies, Carlsbad, CA). The concentrations used in the assays were determined by growth suppression and colony formation assays based on 30-50% of the cells surviving.

2. Growth suppression assay

Ten thousand cells were disseminated in a well of a 96-well microtiter plate. We then added 5-FU over a 10-fold dilution series once the cells reached 80% confluency (range 38.4 mM to 38.4 nM). The concentration of CDDP was maintained at 8.3 μM, which is equivalent to its peak plasma concentration (ppc; 2.5 μg/ml) [10, 11]. For the CODA of 5-FU with CDDP, each drug was maintained in media for 24 hours and then cell growth was measured. Wells with media containing the same respective drug concentrations were also replaced every 12 hours in parallel. For ADDA, the drug was replaced with a drug-free medium every 12 hours for a period of 72 h. For the ADDA of sustained CDDP, either medium containing 5-FU or 5-FU-free medium was replaced every 12 hours. For all administration methods, the 10-fold dilution series of the 5-FU was maintained throughout the assay. Hence, the ADDA group receives 50% of the 5-FU dose in the given time "block". Growth was measured using a Water Soluble Tetrazolium (WST) assay (Dojindo, Mashikimachi, Japan), and the light absorption of the resulting formazan was measured at 450 nm using a multimode plate reader (Berthold Technologies, Germany). The light absorption data were plotted to calculate the 50% growth inhibitory concentration (GI_{50}). Each drug administration procedure is shown in Figure 1A.

3. Cell cycle analysis

For cell cycle analysis, 1 × 10^6 cells were harvested from each well of a six-well plate at 72 h after drug administration. The concentration of 5-FU required to suppress cell growth by 50-60% relative to cells not receiving drug was determined after

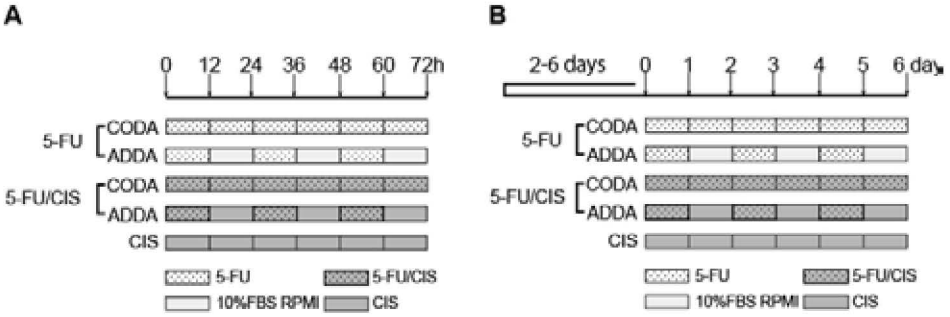

Fig. 1. Drug administration procedures for gastric cancer cell lines.
(A) The growth suppression assay procedure involved six 12-hour time blocks.
(B) The colony formation assay procedure involved six 1-day time blocks.
CIS, CDDP.

administration in the CODA and ADDA groups for each cell line (i.e., GI_{50} to GI_{65}). When CDDP was used, the concentration was adjusted to achieve its ppc for all cell lines. For the combination of 5-FU and CDDP, the 5-FU concentration was determined by the growth suppression assay in the presence of CDDP. The collected cell pellet was analyzed using the Tali® Cell Cycle Kit (Life Technologies), and the distribution of DNA content was promptly calculated using a Tali® Image-based Cytometer (Life Technologies).

4. Colony formation assay

After creating a single-cell suspension of each cell line, the cells were disseminated into a 24-well plate at a density of ～52 cells/cm^2 (i.e., 100 cells/well). After confirming cell attachment, 5-FU was added into the medium as a 10-fold dilution series (range 38.4 mM to 38.4 nM). For CODA, the cells were maintained in the wells until the colonies that formed were at least 0.5 mm in diameter. For ADDA, the medium replacement was repeated every 24 hours until colonies that were at least 0.5 mm in diameter emerged. The concurrent administration of 5-FU and CDDP was also performed using the same procedure as the ADDA and CODA of 5-FU alone, except that the medium always contained 8.5 μM CDDP. The drug-tolerant colonies were stained with 0.1% crystal violet solution in a 50% methanol solution and then captured as an image on an optical flatbed scanner (Epson, Suwa, Japan) with a resolution of 600 dpi for manual counting. Each drug administration procedure is shown in Figure 1B.

5. Apoptosis measurement by Annexin V (ANXV) and propidium iodide (PI)

Cells that were sensitive to 5-FU (MKN7 and MKN74) and insensitive to 5-FU (MKN45 and GCIY) were grown in a 6-well plate under each drug administration condition as described above. After drug treatment, cells were stained using Annexin V Alexa Fluor® 488 and a PI kit followed by measurement on a TaliTM Image-based cytometer (Life Technologies). Apoptotic cells were detected with green fluorescence while necrotic cells were visualized with red fluorescence. After staining, 20 independent visual fields of cells were captured with the TaliTM imaging cytometer to quantify the fluorescence and distribution. The total number of PI-positive and Annexin V-positive cells was counted to determine the apoptotic and necrotic fractions, re spectively [12].

6. Cell invasion and migration assay

A cell invasion assay was performed based on the Boyden chamber principle [13]. Selected cell lines (GCIY, MKN7, MKN45, and MKN74) were grown in T-25 flasks under the respective drug administration conditions (Fig. 1A). After drug administration, cells were trypsinized and subjected to a single cell suspension. The suspended cells were seeded in serum-free media into a 24-well plate containing an 8 μm pore size polycarbonate insert membrane coated with a thin layer of ECMatrix™ (Millipore, Billerica, MA). The cells capable of invading the membrane migrated towards the medium with FBS in the bottom plate while non-invading cells remained in the insert chamber. After 24 hours of incubation, cells in the bottom chamber are lysed and detected with the CyQuant GR® dye (Molecular Probes, Eugene, OR) on a TriStar LB941 fluorescence microplate reader (Berthold, Bad Wildbad, Germany).

The cell migration ability of the MKN7 and MKN45 cell lines was evaluated using a wound healing assay [14]. Cells were grown up to 80% confluent in a 6-well plate under the different drug administration conditions (Fig. 1A). After drug administration, a straight scratch was made with a pipette tip and an image was taken under a microscope. Forty-eight hours later, images of the scratch in the identical position were captured.

7. Western blot analysis

After cells reached approximately 80% confluence, a subset of cell lines (MKN7 and MKN45) was treated with the respective drug administration conditions. Cell pellets were then collected by scraping the plates and washing the cells three times with cold PBS. The cell pellets were then lysed for 10-30 min in Pink Buffer containing 9M urea (Sigma, St. Louis, MO), 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS; Millipore), 2% pH 8.0-10.5 Pharmalyte (GE Healthcare, Piscataway, NJ), and 65 mM DTT (GE Healthcare) [15]. After lysis, the samples were briefly centrifuged and the supernatants were stored at −80℃ for future analysis or immediately resolved on a 4-15% SDS-polyacrylamide linear gradient gel by electrophoresis. Proteins from the gel were then transferred to a nitrocellulose membrane using the iBlot® dry blotting system (Life Technologies) and examined by Ponceau S staining (Sigma) to ensure equal loading. The membrane was then incubated with a SuperG blocking buffer (Grace BioLabs, Bend, OR) followed by incubation with appropriate primary antibodies (anti-p53, DO-1 mouse monoclonal antibody, Thermo Scientific, Waltham, MA; caspase7, rabbit polyclonal Cleaved caspase7 Asp198 antibody, Cell Signaling Technologies, Danvers, MA; γH2AX, rabbit polyclonal H2AX pS139 antibody, Rockland antibodies & assays, Limerick, PA; and Keratin 19, A53-B/A2.26, mouse monoclonal antibody, Thermo Scientific). The membrane was subsequently washed, incubated with secondary antibodies, and then visualized with an ECL Select Western Blot Detection Reagent (GE Healthcare).

III. Results

1. Concentrations required for 50% growth suppression

The average GI_{50} value of 5-FU for the nine tested gastric cancer cell lines was 17.7 μM (range 0.45 to 59.0; Fig. 2A). Given that the ppc of 5-FU was approximately 15.3 μg/

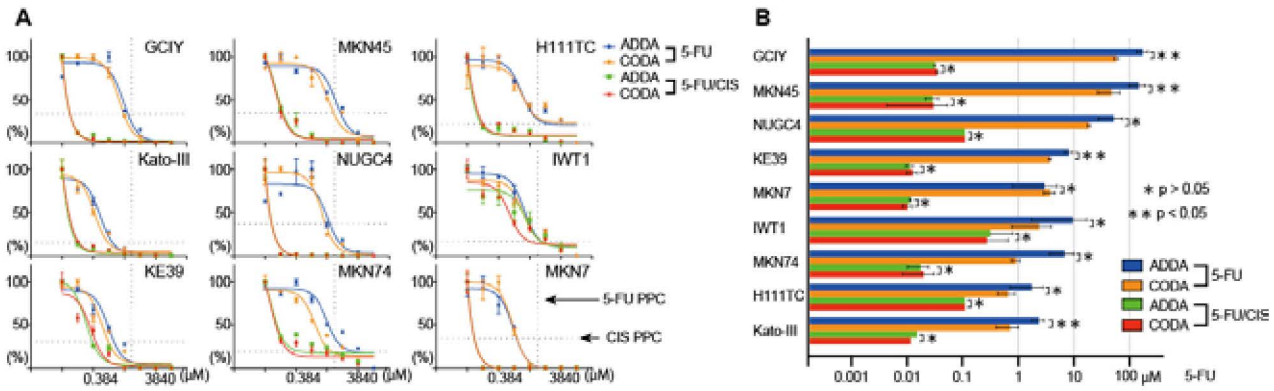

Fig. 2. Growth suppression assay with the CODA and ADDA models in gastric cancer cell lines.
(A) Dose response curves of nine gastric cancer cell lines with four different procedures, including ADDA of 5-FU alone (blue), CODA of 5-FU alone (orange), ADDA of concurrent 5-FU/CDDP (green), and CODA of concurrent 5-FU/CDDP (red). The bar colors are consistent with the dose response curves. The horizontal and vertical axes represent the 5-FU concentration and the growth rate, respectively. The concentration of CDDP remains constant for all 5-FU concentrations. A horizontal broken line shows the ppc of CDDP. A vertical broken line shows the ppc of 5-FU. (B) Bar graphs of the GI_{50} across the nine gastric cancer cell lines. The horizontal axis represents the 5-FU concentration. Each bar represents the GI_{50} of the corresponding drug administration procedure. The indicated p-values were calculated by a Student's *t*-test.

ml (i.e., 117 μM, [16]), the GI_{50} of each cell line is likely to be reached in the human body and is effective for growth suppression. The majority of the cell lines tended to show better growth suppression effects using CODA than with ADDA, although less than half of the cell lines demonstrated a statistically significant difference (Fig. 2B). To observe the combination effect with CDDP, the concentration of CDDP was set to 8.3 μM, which is equivalent to its ppc (2.5 μg/ml) [10, 11]. The baseline growth suppression effect obtained with 8.3 μM CDDP alone was slightly better than 50% (15.0-36.7%), whereas the GI_{50} of 5-FU in combination with CDDP decreased three orders of magnitude (i.e., the GI_{50} decreased >10-10^3 times) for all cell lines in both the CODA and ADDA models (Fig. 2B). Moreover, none of the cell lines subjected to the 5-FU and CDDP combination treatment demonstrated a statistically significant difference in the GI_{50} between the ADDA and CODA models (Fig. 2B). In order to ensure that the CDDP dose used was not too high, we further confirmed that the combinational effect obtained through the CODA decreased to 0.01% of the CDDP ppc (0.083 μM) in five of the cell lines: MKN45, NUGC-4, KE39, IWT-1, and Kato-III. These observations suggest that ADDA could be a substitute for CODA in a majority of gastric cancer cases, particularly when combined with CDDP.

2. Colony formation in the presence of antitumor drugs

With the exception of MKN7 and H111TC, all of the cells were capable of forming colonies. Two cell lines, GCIY and NUG4, showed differences under ADDA and CODA, whereas the remaining five cell lines (Kato-III, KE39, MKN74, IWT-1, and MKN45) showed a similar degree of colony formation under both ADDA and CODA models (Fig. 3A). In

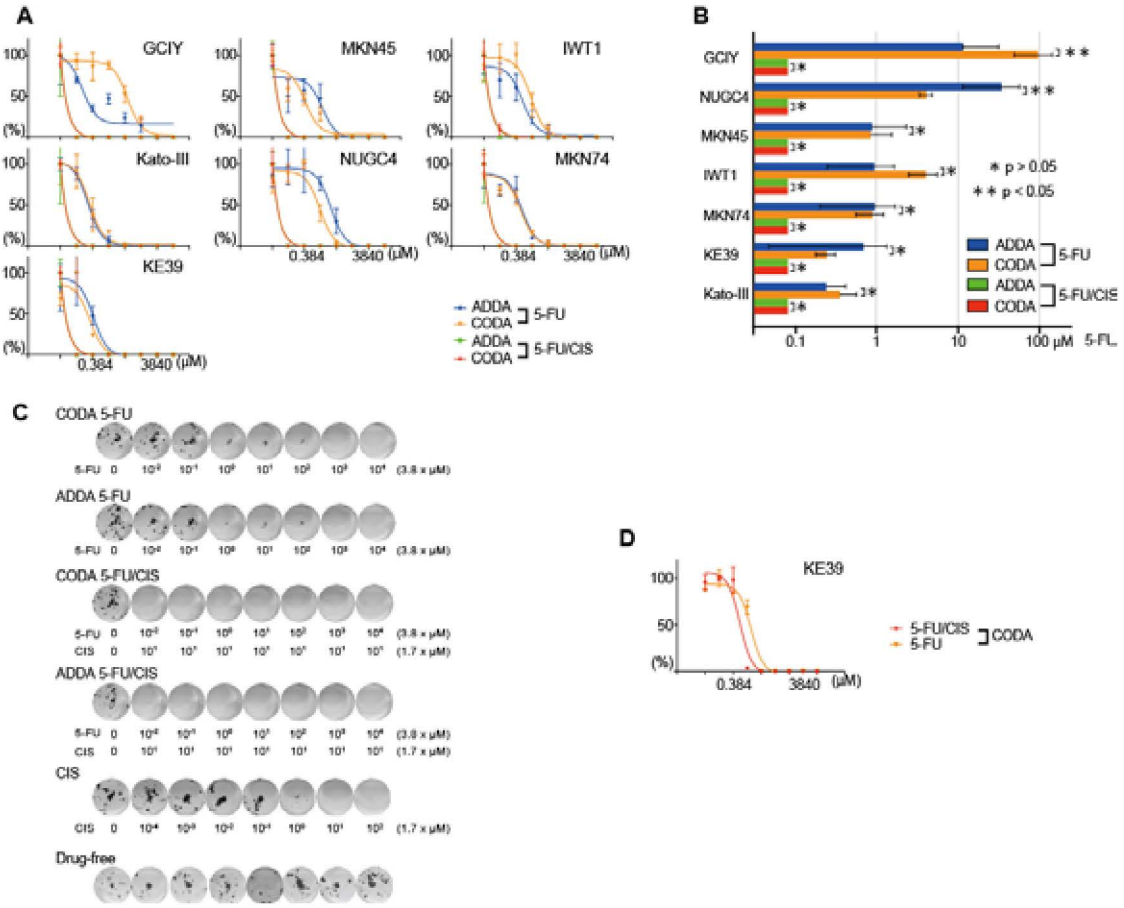

Fig. 3. Colony formation assay for the CODA and ADDA models in gastric cancer cell lines.

(A) Dose response curves of seven gastric cancer cell lines obtained with four different procedures: ADDA of 5-FU alone (blue), CODA of 5-FU alone (orange), ADDA of concurrent 5-FU/CDDP (green), and CODA of concurrent 5-FU/CDDP (red). The horizontal and vertical axes represent the 5-FU concentration and the colony formation rate, respectively. The concentration of CDDP remains constant for all 5-FU concentrations.

(B) Bar graphs of CI_{50} across seven gastric cancer cell lines. The horizontal axis represents the 5-FU concentration. Each bar represents the CI_{50} of the corresponding drug administration procedure. The bar colors are consistent with the dose response curves. The indicated p-values were calculated by the Student's *t*-test.

(C) Representative images of the colony formation of MKN74 cells after ADDA and CODA. The dilution series of the 5-FU concentration is constant in the following procedures: CODA of 5-FU alone, ADDA of 5-FU alone, CODA of concurrent 5-FU/CDDP, ADDA of concurrent 5-FU/CDDP, CODA of CDDP alone, and drug-free colonies. The drug concentration in each well is indicated below the respective colony image. The colonies were stained with 0.1% crystal violet.

(D) Dose response curves of the colony formation of MKN45 and KE39 cells after the CODA of 5-FU alone and CODA of concurrent 5-FU/CDDP with 0.01% of the CDDP ppc. The horizontal and vertical axes represent the 5-FU concentration and the colony formation rates, respectively.

CIS, CDDP.

the presence of CDDP at ppc, the degrees of colony formation were all similar for both ADDA and CODA models (Fig. 3B). However, it should be noted that 8.5 μM CDDP did not completely suppress colony formation when CDDP was administered alone (Fig. 3C).

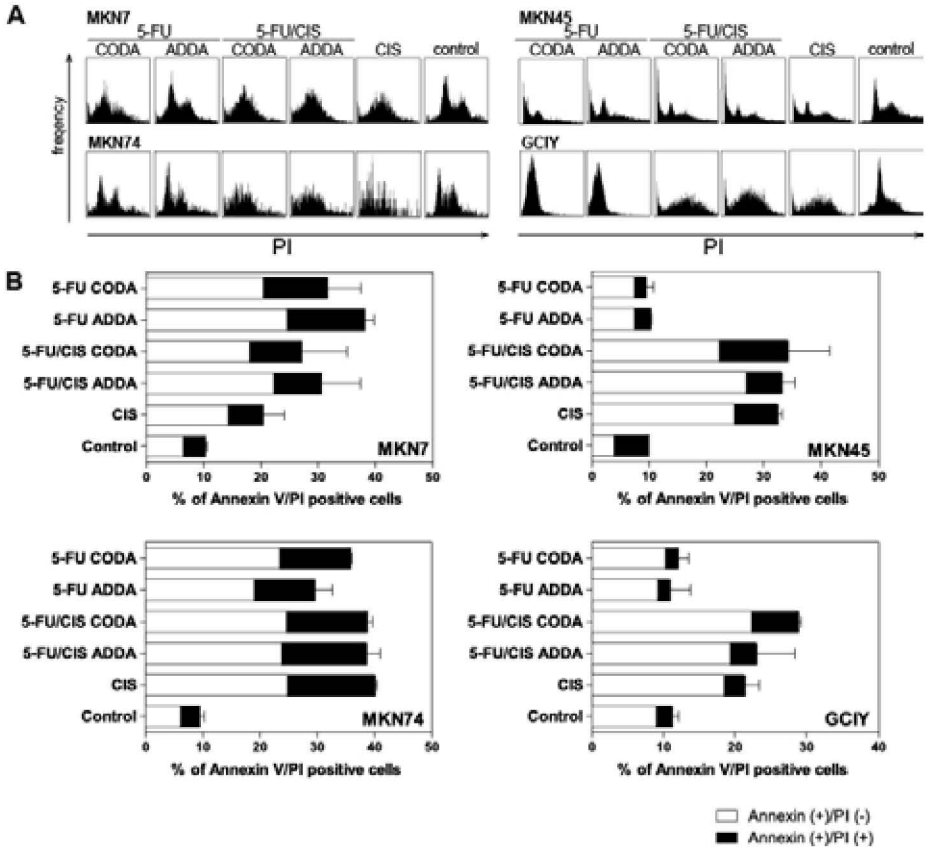

Fig. 4. Monitoring of cell death-related fractions.
(A) The name of the cell line is shown on the top left corner of each set of panels. The horizontal and vertical axes represent the strength of the PI staining and the frequency, respectively. Each panel contains six experiments indicated on top of each panel. PI, propidium iodide.
(B) Drug-induced "cell death" fractions stained with Annexin V and PI. Annexin V and PI levels were measured by an imaging cytometer and quantified in four cell lines. MKN7 and MKN74 are 5-FU-sensitive cell lines, whereas MKN45 and GCIY are 5-FU-insensitive cell lines based on GI_{50} value indicated in Figure 2. White bars indicate Annexin V-positive/PI-negative fractions. Black bars indicate Annexin V-positive/PI-positive fractions. Error bars indicate standard error of the mean of the sum of Annexin V-positive/PI-negative and Annexin V-positive/PI-positive fractions. CIS, CDDP.

Although CDDP appears to be more influential in colony suppression than 5-FU when 5-FU and CDDP are administered simultaneously, the results from treatment with 8.5 μM CDDP alone indicate a combinational effect of 5-FU and CDDP. We also investigated the suppression of colony formation under the CDDP concentration as low as 0.01% of the ppc in KE39 cells. Under this condition, we still found that the combination of drugs enhanced the inhibition of colony formation (Fig. 3D).

3. Effect on the cell cycle by different drug administration procedures

The cell cycle distributions did not show substantial differences between the CODA and ADDA models of 5-FU (Fig. 4A). The effects of 5-FU were slightly different depending on the cell lines, including G1 arrest (GCIY), S/G2 arrest (MKN74), and an increase of sub-G1

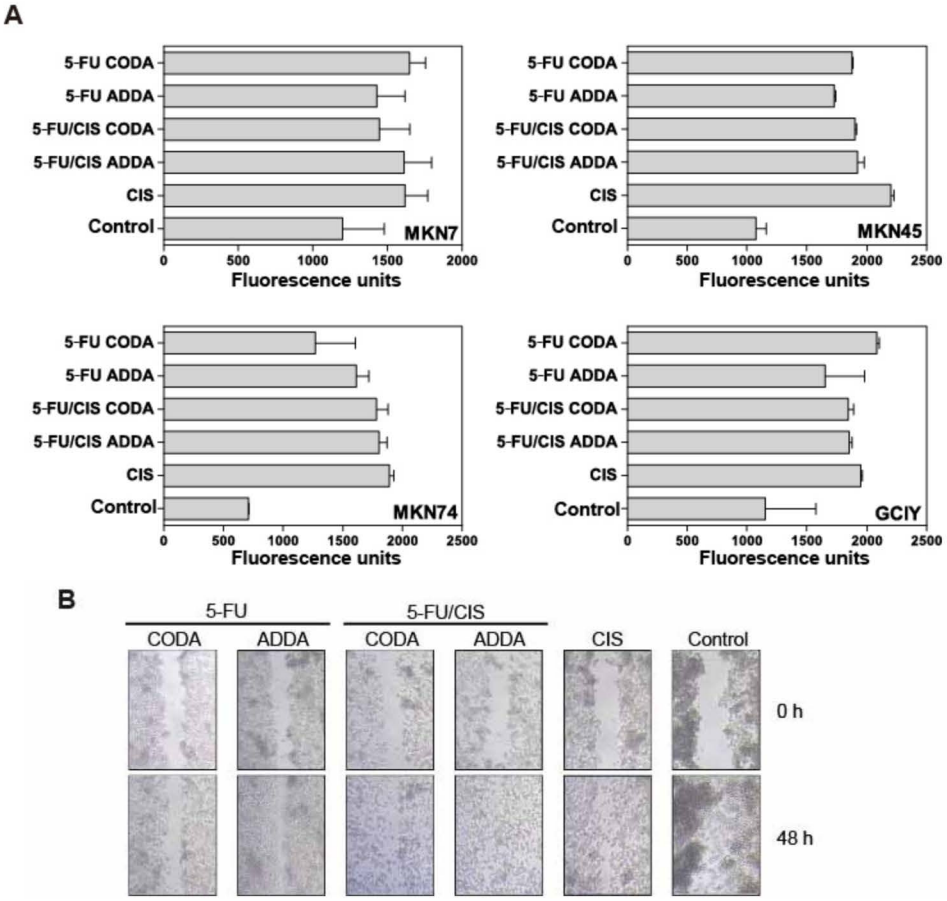

Fig. 5. Effect on the ability of cellular invasion and migration by 5-FU and CDDP.
(A) Each bar length represents the fluorescence strength reflecting the immobilized cell number in an insert chamber. The name of the cell line is indicated at the right bottom corner of each panel. Experiments for each condition were repeated at least three times. Error bars indicate standard error of the mean.
(B) Images of wound healing assay after completing each drug administration procedure (0 hour) and 48 hours later for the MKN45 cell line. The drug administration is indicated at the top of each image.
CIS, CDDP.

fractions (MKN45 and MKN7). The continuous administration of CDDP alone induced an increase of the sub-G1 fractions for all of the cell lines. The combination of 5-FU and CDDP also exhibited sub-G1 distributions, but these were not always identical to those obtained after single agent administration. These cell cycle distributions indicated that the type of drug used was the most influential factor on cell cycle distribution, rather than the drug administration mode.

4. Cell death induced by ADDA and CODA

Drugs such as 5-FU and CDDP can induce two major mechanisms of cell death. Apoptosis is considered to be the predominant mechanism for cell death, which can occur as a result of genotoxic agent administration, whereas necrosis is caused by the physiological damage of cells, such as swollen organelles and plasma membrane rupture [17]. Although the major consequence of antitumor therapeutics is apoptosis, necrosis has been recognized as

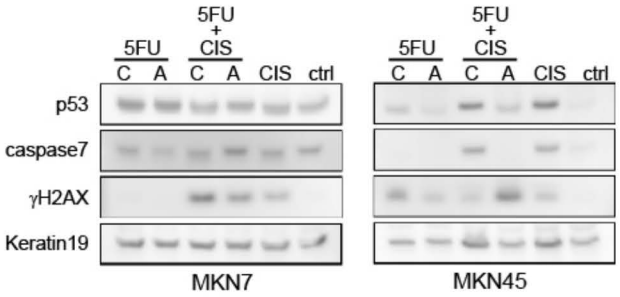

Fig. 6. Drug-induced changes in protein levels as monitored by Western blot.
The cell lysate obtained from each drug administration condition is indicated at the top. The proteins probed with primary antibodies are indicated on the left. C, CODA; A, ADDA; and ctrl, control. Keratin 19 was used as a loading control.
CIS, CDDP.

another important mechanism for inducing cell death in response to cellular stress [18, 19].

We found that the $ANXV^+/PI^-$ fractions accounted for less than 10% of the total cell fraction for any of the administration conditions, which was not entirely consistent with the corresponding sub-G1 fractions (Fig. 4AB). This is perhaps partially due to a direct effect of the genotoxic agents, which resulted in immediate cell death before the cells could undergo apoptosis [20]. Therefore, we measured the necrotic fractions (i.e., $ANXV^+/PI^+$) as well to determine the sum of the "cell death" fractions. The total cell death fractions were at least 2-3 times higher for all combined administration methods in 5-FU sensitive MKN7 and MKN74 cell lines (Fig. 4B). In contrast, the 5-FU insensitive MKN45 and GCIY lines demonstrated a clear combination effect of 5-FU and CDDP (Fig. 4B). Based on these experiments, the following two major conclusions were drawn: (a) The ADDA and CODA models have an equivalent degree of antitumor effect in 5-FU sensitive cell lines; and (b) the combination of CDDP and 5-FU may increase the induction of cell death by up to 40% in both ADDA and CODA models.

5. Effect of antitumor drugs on cellular invasion and migration

One of the major forms of post-operative gastric cancer recurrence is metastasis, and an invasion and migration phenotype *in vitro* is closely associated with the potential of metastasis [21]. All cell lines tested showed significant suppression of invasion for both drug administration models (Fig. 5A). No remarkable difference in the invasion ability was observed between the ADDA and CODA models, presence or absence of CDDP, or cell sensitivity to 5-FU. We also assessed the cell migration ability by using a wound healing assay (Fig. 5B). MKN45 in drug-free medium occasionally demonstrated an expansion in the vertical axis. Cells in drug-free medium spread within the scratched line faster than those treated with drugs, and also exhibited cellular proliferation. Both ADDA and CODA suppressed the migration. Interestingly, ADDA with CDDP and CODA with CDDP seemed to show better migration than those without CDDP. In principle, the measured abilities of invasion and migration were highly concordant, particularly for the MKN45 cell line. These results suggest that ADDA is sufficient for suppressing gastric cancer cell invasion and migration relative to CODA.

6. Monitoring of molecular responses

To investigate the type of molecular responses that occur after the two drug administration models, the protein expression levels of p53, caspase7, and γH2AX were examined by western blot in MKN7 and MKN45 cells (Fig. 6). The p53 levels of MKN7

were slightly increased in both the ADDA and CODA models when 5-FU was administrated alone. Although caspase7 was slightly increased in the ADDA model with CDDP in MKN7, it did not directly correlate with the corresponding apoptotic fraction (Fig. 4). The levels of γH2AX were elevated in response to CDDP with or without 5-FU in MKN7 cells, suggesting that CDDP exclusively induces a DNA damaging effect. In MKN45, both p53 and caspase7 levels were increased in the CODA model with CDDP alone and in combination with CDDP, indicating that continuous CDDP administration may be an effective means for inducing apoptosis in a p53-dependent manner. There was also a clear induction of γH2AX expression in MKN45 cells after 5-FU treatment alone in the CODA model as well as with CDDP in the ADDA model.

IV. Discussion

The design of ADDA in the present study consisted of a 12-hour medium replacement cycle instead of an "every other day" method based on the regimen currently used in the clinic [6, 22]. Tumor doubling time is associated with the duration of the S- phase, for which only 5-FU is effective, whereas the ADDA model is a regimen in which each drug-free interval is shorter than the tumor doubling time in humans [23]. Since normal cells (e.g., gastrointestinal mucosa and bone marrow cells) generally have a shorter S-phase (about 10 h) than tumor cells (17-60 hours), ADDA can still sustain antitumor activity while concomitantly reducing the adverse effects [5, 22, 24]. In the present cell line panel, the median doubling time of the cell lines was 37 hours. If 20-40% of the cell cycle is S-phase [25], the 12-hour interval in the present ADDA model should expose 5-FU long enough to be in-taken, which would provide relevant information for antitumor effect.

Clinically, a treatment is considered effective if a tumor stays at equilibrium (i.e., "Stable Disease" in RECIST, [26]), whereas growth suppression *in vitro* is a simple result of how many cells survive relative to the control. The most clinically relevant finding from the present study is that ADDA is equivalent to CODA in terms of growth suppression when CDDP is continuously administered. In the presence of CDDP, it can be postulated that the cell cycle of the cancer cells slows down, since exposed cells remain in the S/G2 phase longer. Therefore, differences between the ADDA and CODA of 5-FU diminish [5, 27]. In terms of colony formation, 5-FU demonstrated effective suppression in the presence of CDDP at its ppc. Moreover, 0.01% of the ppc of CDDP also showed a colony inhibitory effect when combined with 5-FU. Some cases of post-curative surgery of advanced cancer experience recurrence, suggesting that undetectable cancer cells still remain in the body. The cancer cells occasionally form metastatic lesions or dissemination in the peritoneum, in which cancer cells seem to propagate from small colonies even in the presence of chemotherapeutic agents. Since cancer cells are disseminated at an undetectable density for colony formation, this assay provides a good model of recurrent tumors after chemotherapy [28]. CDDP alone does not always completely suppress cancer recurrence in practice. Therefore, these results suggest that ADDA combined with even low-dose CDDP can effectively suppress colony formation, which might repress

recurrence [29].

We also investigated the cellular and molecular mechanisms that may be responsible for the differences in the degree of antitumor effects between the ADDA and CODA models. Profiles of cell cycle distribution demonstrated that 5-FU and CDDP seemed to cause the cellular consequences unique to drug combination, rather than administration regimens. These observations suggest that ADDA and CODA have no remarkable differences in terms of the effect on cell cycle distribution. We further validated the "cell death" fraction induced by ADDA and CODA by means of Annexin V and PI staining. The cell death fractions observed after treatment were also dependent on the drug type, but not on the administration regimen. Rather, differences between the ADDA and CODA models appeared to be cell line-dependent. The 5-FU-sensitive cell lines (MKN7 and MKN74) showed 20-40% induction of cell death under both administrative conditions, whereas the 5-FU-insensitive cell lines (MKN45 and GCIY) showed almost no induction of cell death when 5-FU was administrated alone. However, the combination of 5-FU and CDDP induced significant cell death in the 5-FU-insensitive cell lines. Moreover, the invasion assay indicated that drug type seemed to be the most important factor for suppressing the migration/invasion ability, rather than the drug administration model. These results suggest the following two possibilities: (a) ADDA and CODA models are equally effective in terms of antitumor effect, and (b) continuous CDDP administration on top of any 5-FU administration increases the sensitivity of 5-FU-insensitive cells. In practice, these results also support the hypothesis that ADDA may induce comparable antitumor effects to CODA with a lower chance of adverse effects [30].

To support these phenotypic findings, cellular responses to the drugs at the molecular level were also investigated. Both MKN7 and MKN45 cells possess a wild-type TP53 gene, although the p53 protein levels are relatively low under non-stressed conditions [9]. We found that the stress marker p53 was stabilized in MKN7 under almost all conditions [31], while CDDP alone and CODA with CDDP seemed to increase p53 levels in MKN45. Caspase7 levels did not seem to be directly indicative of response in MKN7 cells. However, a similar increasing trend of caspase7 and p53 levels in MKN45 cells suggests that CDDP and CODA may have activated a p53-regulated apoptosis pathway [32]. We also assessed γH2AX expression as a biomarker for chemotherapeutics that induce DNA damage [33]. We found that the continuous administration of CDDP was sufficient to induce DNA damage in a 5-FU-sensitive cell line (MKN7), while DNA damage was induced by 5-FU alone in a 5-FU-insensitive cell line (MKN45). The activation of the apoptosis pathway did not seem to be directly associated with DNA damage and 5-FU sensitivity. Together with the result of the cell death fraction experiment, these results suggest that there may be multiple molecular pathways for inducing cell death.

Our present study demonstrated that the antitumor effect of 5-FU was similar in both the ADDA and CODA models in human gastric cancer cell lines. In addition, continuous

CDDP administration appeared to increase the antitumor effect of 5-FU in 5-FU insensitive cells. However, the molecular signals that occur in response to drug administration may be different depending on individual cells, suggesting that the cell death pathway may vary in a cell-dependent manner. Therefore, the addition of 5-FU modulators should be considered carefully depending on the molecular profile of individual tumors. In conclusion, our *in vitro* findings suggest that ADDA may be a reasonable alternative regimen to CODA and provides similar antitumor effects while possibly minimizing adverse effects of S-1.

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References

1) **Shirasaka T, Shimamato Y, Ohshimo H, et al.**: Development of a novel form of an oral 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor selective cytotoxicity of 5-fluorouracil by two biochemical modulators. Anticancer Drugs **7**, 548-557, 1996.
2) **Sasako M, Sakuramoto S, Katai H, et al.**: Five-year outcomes of a randomized phase III trial comparing adjuvant chemotherapy with S-1 versus surgery alone in stage II or III gastric cancer. J Clin Oncol **29**, 4387-4393, 2011.
3) **Sakuramoto S, Sasako M, Yamaguchi T, et al.**: Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. N Engl J Med **357**, 1810-1820, 2007.
4) **Tatebe S, Tsujitani S, Nakamura S, et al.**: Feasibility study of alternate-day S-1 as adjuvant chemotherapy for gastric cancer: a randomized controlled trial. Gastric cancer (Official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association) **17**, 508-513, 2014.
5) **Shirasaka T**: Development history and concept of an oral anticancer agent S-1 (TS-1): its clinical usefulness and future vistas. Jpn J Clin Oncol **39**, 2-15, 2009.
6) **Arai W, Hosoya Y, Haruta H, et al.**: Comparison of alternate-day versus consecutive-day treatment with S-1: assessment of tumor growth inhibition and toxicity reduction in gastric cancer cell lines in vitro and in vivo. Int J Clin Oncol **13**, 515-520, 2008.
7) **Ishida K, Nishizuka SS, Chiba T, et al.**: Molecular marker identification for relapse prediction in 5-FU-based adjuvant chemotherapy in gastric and colorectal cancers. PLoS One **7**, e43236, 2012.
8) **Tsuji A, Shima Y, Morita S, et al.**: Combination chemotherapy of S-1 and low-dose twice-weekly cisplatin for advanced and recurrent gastric cancer in an outpatient setting: a retrospective study. Anticancer Res **28**, 1433-1438, 2008.
9) **Endo F, Nishizuka SS, Kume K, et al.**: A compensatory role of NF-kappaB to p53 in response to 5-FU-based chemotherapy for gastric cancer cell lines. PLoS One **9**, e90155, 2014.
10) **Ikeda K, Terashima M, Kawamura H, et al.**: Pharmacokinetics of cisplatin in combined cisplatin and 5-fluorouracil therapy: a comparative study of three different schedules of cisplatin administration. Jpn J Clin Oncol **28**, 168-175, 1998.
11) **Kawamura H, Ikeda K, Takiyama I, et al.**: The usefulness of the ATP assay with serum-free culture for chemosensitivity testing of gastrointestinal cancer. Eur J Cancer **33**, 960-966, 1997.
12) **Schulze-Lohoff E, Hugo C, Rost S, et al.**: Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via P2Z/P2X7 receptors. Am J Physiol **275**, F962-971, 1998.
13) **Boyden S**: The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J Exp Med **115**, 453-466, 1962.
14) **Liang CC, Park AY** and **Guan JL**: In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat

Protoc **2**, 329-333, 2007.
15) **Nishizuka S, Charboneau L, Young L, et al.**: Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. Proc Natl Acad Sci USA **100**, 14229-14234, 2003.
16) **Casale F, Canaparo R, Serpe L, et al.**: Plasma concentrations of 5-fluorouracil and its metabolites in colon cancer patients. Pharmacol Res **50**, 173-179, 2004.
17) **Xu Y, Lin Z, Zhao N, et al.**: Receptor interactive protein kinase 3 promotes Cisplatin-triggered necrosis in apoptosis-resistant esophageal squamous cell carcinoma cells. PLoS One **9**, e100127, 2014.
18) **Chen B, Xu M, Zhang H, et al.**: Cisplatin-induced non-apoptotic death of pancreatic cancer cells requires mitochondrial cyclophilin-D-p53 signaling. Biochem Biophys Res Commun **437**, 526-531, 2013.
19) **Vaseva AV, Marchenko ND, Ji K, et al.**: p53 opens the mitochondrial permeability transition pore to trigger necrosis. Cell **149**, 1536-1548, 2012.
20) **Kravchenko-Balasha N, Mizrachy-Schwartz S, Klein S, et al.**: Shift from apoptotic to necrotic cell death during human papillomavirus-induced transformation of keratinocytes. J Biol Chem **284**, 11717-11727, 2009.
21) **El-Merahbi R, Liu YN, Eid A, et al.**: Berberis libanotica ehrenb extract shows anti-neoplastic effects on prostate cancer stem/progenitor cells. PLoS One **9**, e112453, 2014.
22) **Terashima M, Irinoda T, Kawamura H, et al.**: Intermittent FLDP: 24-h infusion of 5-FU on days 1, 3 and 5 combined with low-dose cisplatin on days 1-5 for gastric cancer, and its pharmacologic and kinetic rationale. Cancer Chemother Pharmacol **51**, 240-246, 2003.
23) **Stathopoulou A, Roukos V, Petropoulou C, et al.**: Cdt1 is differentially targeted for degradation by anticancer chemotherapeutic drugs. PLoS One **7**, e34621, 2012.
24) **Saga Y, Suzuki M, Sato I, et al.**: An in vitro examination of a 5-fluorouracil regimen involving continuous venous infusion using cultured cell lines derived from ovarian cancers. Oncol Rep **7**, 625-628, 2000.
25) **Barranco SC, Ford PJ** and **Townsend CM, Jr.**: Cell cycle kinetics responses of human stomach cancer cells to reduction in polyamine levels by treatment with alpha difluoromethylornithine in vitro. Invest New Drugs **4**, 347-357, 1986.
26) **Nishino M, Jagannathan JP, Ramaiya NH, et al.**: Revised RECIST guideline version 1.1: What oncologists want to know and what radiologists need to know. AJR Am J Roentgenol **195**, 281-289, 2010.
27) **Yamaue H, Satoi S, Kanbe T, et al.**: Phase II clinical study of alternate-day oral therapy with S-1 as first-line chemotherapy for locally advanced and metastatic pancreatic cancer. Cancer Chemother Pharmacol **73**, 97-102, 2014.
28) **Matsuo T, Nishizuka SS, Ishida K, et al.**: Evaluation of chemosensitivity prediction using quantitative dose-response curve classification for highly advanced/relapsed gastric cancer. World J Surg Oncol **11**, 11, 2013.
29) **Kunisaki C, Ono HA, Hasegawa S, et al.**: Low-dose docetaxel and cisplatin combination chemotherapy for stage II/III gastric cancer showing resistance to S-1 adjuvant chemotherapy: a phase I study. J Chemother **24**, 364-368, 2012.
30) **Arai W, Hosoya Y, Hyodo M, et al.**: Alternate-day oral therapy with TS-1 for advanced gastric cancer. Int J Clin Oncol **9**, 143-148, 2004.
31) **Berkers CR, Maddocks OD, Cheung EC, et al.**: Metabolic regulation by p53 family members. Cell Metab **18**, 617-633, 2013.
32) **Sun M, Zhou T, Jonasch E, et al.**: DDX3 regulates DNA damage-induced apoptosis and p53 stabilization. Biochim Biophys Acta **1833**, 1489-1497, 2013.
33) **Ivashkevich A, Redon CE, Nakamura AJ, et al.**: Use of the gamma-H2AX assay to monitor DNA damage and repair in translational cancer research. Cancer Lett **327**, 123-133, 2012.
34) **Nozue M, Nishida M, Todoroki T, et al.**: Establishment and characterization of a human scirrhus type gastric cancer cell line, GCIY, producing CA19-9. Hum Cell **4**, 71-75, 1991.
35) **Uesugi H, Yamashita K, Atari E, et al.**: Establishment and characterization of strain KE-39 derived from human gastric cancer. Hum Cell **7**, 227-232, 1994.
36) **Sekiguchi M, Sakakibara K** and **Fujii G**: Establishment of cultured cell lines derived from a human gastric carcinoma. Jpn J Exp Med **48**, 61-68, 1978.
37) **Wong J, Kelly K, Mittra A, et al.**: A third-generation herpesvirus is effective against gastroesophageal cancer. J Surg Res **163**, 214-220, 2010.
38) **Akiyama S, Amo H, Watanabe T, et al.**: Characteristics of three human gastric cancer cell lines, NU-GC-2, NU-GC-3 and NU-GC-4. Jpn J Surg **18**, 438-446, 1988.

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ヒト胃癌細胞を用いた 5-FU 隔日投与の抗腫瘍効果の評価

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要旨

胃癌の化学療法として 5-FU をベースとしたレジメが確立されている．しかしながら，副作用のため標準治療を完遂できない患者が相当数認められる．経口フルオロピリミジン剤である S-1 の抗腫瘍効果を損なわずに副作用の発症を下げる試みとして，隔日投与法が提唱されている．しかしながら，その抗腫瘍効果については一定の見解は得られていない．本研究では，隔日投与のモデルとして，ヒト胃癌細胞株 9 種類を用いた 12 時間の間歇投与および連続投与による，増殖抑制試験，コロニー形成試験，細胞周期測定を行った．薬剤投与は，5-FU 単独あるいは 5-FU とシスプラチンの併用を用いた．大部分の細胞株で間歇投与と連続投与の抗腫瘍効果は同等であり、シスプラチンと 5-FU を併用した場合には 9 種類全てでその効果の同等性が認められた．以上のことから，5-FU の隔日投与により抗腫瘍効果を損なわずに治療可能であることが示唆された．