The Combination of 5-FU, Leucovorin and CPT-11 (FOLFIRI) Prolongs Survival through Inhibition of Metastasis in an Orthotopic Model of Colon Cancer

YOKO ISHIHARA[1], KENICHI MATSUNAGA[2], HIROKO IIJIMA[2], GO HASEGAWA[1], TOSHIMITSU SUZUKI[3], ATSUSHI SATO[4], TOMOWO KOBAYASHI[5], MENG YANG[6] and ROBERT M. HOFFMAN[6,7]

[1]*Department of Public Health, School of Medicine, Kurume University, Kurume, Fukuoka 830-0011, Japan;*
[2]*Biomedical Research Laboratories, Kureha Corporation, Hyakunincho, Shinjuku, Tokyo 169-8503, Japan;*
[3]*Department of Pathology, Fukushima Medical University School of Medicine, Hikarigaoka, Fukushima, Fukushima 960-1295, Japan;*
[4]*Department of Medical Oncology, Showa University Hospital, Hatanodai, Shinagawa, Tokyo 142-8555, Japan;*
[5]*Foundation for Drug Discovery, Pharma Valley Center, Hamamatsu, Shizuoka 430-8558, Japan;*
[6]*AntiCancer Inc., San Diego, CA 92111, U.S.A.;*
[7]*Department of Surgery, University of California, San Diego, CA 92103, U.S.A.*

Abstract. *The aim of this study was to determine the antimetastatic efficacy of the combination of 5-fluorouracil (5-FU), leucovorin (LV), and irinotecan (CPT-11) (FOLFIRI) in an orthotopic nude mouse model of GFP-HCT-116 human colon cancer-expressing green fluorescent protein (GFP). The mice were randomly divided into four groups: Group 1: saline control; Group 2: 30 mg/kg 5-FU + 90 mg/kg LV; Group 3: 5-FU + LV + 16 mg/kg CPT-11 (FOLFIRI); and Group 4: 5-FU + LV + 24 mg/kg CPT-11. 5-FU and LV were administered on days 11, 16 and 21, and CPT-11 on days 12, 18 and 22. Survival in Groups 3 and 4 was significantly longer than that in Groups 1 and 2, although no dose-dependency on CPT-11 was observed. Analysis of the primary and metastatic tumors by GFP imaging, as well as that of oncogene expression in mesentery lymph nodes, demonstrated that tumor growth and metastasis were significantly inhibited or even prevented by FOLFIRI. Pathological evaluation also demonstrated that metastasis was also inhibited by FOLFIRI. The results of the present study suggest FOLFIRI prolongs survival by inhibiting metastasis.*

Outcomes in patients with advanced colon cancer have been significantly improved by recent advances in chemotherapy, including biochemical modulation and the development of novel anticancer agents (1, 2). A combination regimen of 5-fluorouracil (5-FU) and leucovorin (LV) along with irinotecan hydrochloride (CPT-11), termed "FOLFIRI", is now widely used as a therapy for colon cancer. High response rates and increased survival times for this regimen have been reported, based on randomized controlled trials for colon cancer carried out in the United States and Europe (3, 4). However, it is not clear whether this particular drug combination prolongs survival through inhibition of primary tumor growth, or through suppression of metastasis, or both.

Among the agents used in FOLFIRI, 5-FU inhibits cancer cell proliferation by interfering with DNA synthesis and RNA function (5). While having no anticancer activity itself, LV enhances the activity of 5-FU (6). CPT-11 also inhibits the proliferation of cancer cells and causes tumor remission by inhibiting topoisomerase, an enzyme necessary for the replication and transcription of DNA (7). Although the anticancer efficacy of FOLFIRI is believed to result from the synergetic or additive effects of multiple agents with different mechanisms of action, the exact details remain to be elucidated. The aim of the present study was to determine the antitumor and antimetastatic efficacy of FOLFIRI in a clinically-relevant nude-mouse model of colon cancer. This model enabled the investigation of the mechanism by which FOLFIRI prolongs survival.

Materials and Methods

Drug administration. 5-FU, LV and CPT-11 were either purchased from or provided by Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan), Wyeth K. K. (Tokyo, Japan), and Yakult Honsha Co., Ltd. (Tokyo,

Correspondence to: Yoko Ishihara, Department of Public Health, School of Medicine, Kurume University, Asahimachi, Kurume, Fukuoka 830-0011, Japan. e-mail: blackcat@med.kurume-u.ac.jp

Key Words: Orthotopic model, FOLFIRI, colon cancer, tumor-growth metastasis, survival time, GFP imaging.

Japan) respectively. These agents were adjusted to the administered doses with saline immediately before use. The dose and schedule of 5-FU and LV administration were preliminarily tested based on reports by Tschmelitsch *et al*. (8) and Behr *et al*. (9). The mice were randomly divided into four groups: Group 1: saline control; Group 2: 30 mg/kg 5-FU + 90 mg/kg LV; Group 3: 5-FU + LV + 16 mg/kg CPT-11; and Group 4: 5-FU + LV + 24 mg/kg CPT-11. Each group consisted of 7-9 mice. LV was intraperitoneally administered at 50 mg/kg on days 11, 16 and 21 after tumor transplantation, followed one hour later by intravenous administration of 5-FU at 30 mg/kg. CPT-11 was administered intravenously on days 12, 18 and 22 after tumor transplantation at a dose of either 18 mg/kg or 24 mg/kg. In the control group, saline was administered in the same manner, at a dose of 0.2 ml/20 g weight.

Green fluorescent protein (GFP) gene transduction of HCT-116 human colon cancer cells.

Production of GFP retrovirus. The pLEIN retroviral vector (Clontech Laboratories, Inc., Palo Alto, CA, USA), expressing GFP and the neomycin resistance gene on the same bicistronic message, was used as a GFP expression vector. PT67, an NIH3T3-derived packaging cell line, expressing the 10 Al viral envelope, was purchased from Clontech Laboratories, Inc. PT67 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, Calabasas, CA, USA). For vector production, packaging cells (PT67) at 70% confluence were incubated with a precipitated mixture of DOTAP Liposomal Transfection Reagent *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl-sulfate (DOTAP) reagent (Boehringer Mannheim, Indianapolis, IN, USA). Saturating amounts of pLEIN plasmid were added for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 h after transduction. For selection, the cells were cultured in the presence of 500-2000 μg/ml G418 (Life Technologies, Inc., Grand Island, NY, USA) for 7 days to select for a clone producing high amounts of GFP retroviral vector (PT67-GFP) (10).

GFP gene transduction of cancer cells. For GFP gene transduction, 70% confluent cultures of cancer lines were used. Cancer cells were incubated for 72 h with a 1:1 precipitated mixture of retroviral supernatants of PT67-GFP cells and RPMI-1640 (Fisher Scientific, Santa Ana, CA, USA) containing 10% FBS. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 h after transduction and subcultured at a ratio of 1:15 in selective medium, which contained 200 μg/ml G418. The level of G418 was increased stepwise up to 800 μg/ml. GFP-expressing cancer cells were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, USA) using trypsin/EDTA and amplified by conventional culture methods (11).

Orthotopic transplantation. Nude mice, 6 to 8 weeks old, were used. The mice were anesthetized prior to surgery with a ketamine mixture (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl of acepromazine maleate and 10 μl H_2O) *via s.c.* injection. Fifty microliters containing 2×10^6 HCT-116-GFP cells per mouse were injected into the subcutaneous tissue using a 0.5 ml 28 G latex-free syringe (TYCO Health Group LP, Mansfield, MA, USA). After subcutaneous tumor growth, tumor fragments (1 mm^3), stably expressing GFP, harvested from the resulting *s.c.* tumors in the nude mice, were implanted by surgical orthotopic implantation on the colon of nude mice. After proper exposure of the colon through a lower-left abdominal incision, 8-0 surgical sutures were used to attach the tumor pieces under the serosa of the ascending colon (7). The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer (12).

Animal care. The mice were housed in polycarbonate cages (L 22 cm × H 132 cm × H 135 cm) with sterilized sawdust on the floor and allowed free access to CE-2 feed (Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized tap water. Each cage contained 3-5 mice. The environment was kept at 25±2˚C, with 55±10% humidity, and a luminous intensity of 5 lx, with a light cycle from 8:00 AM to 8:00 PM under laminar air flow. The weight of the transplanted animals was checked regularly. Autopsy was performed to determine and record metastatic status, as well as cause of death.

Whole-body imaging of tumor growth and metastasis. On days 35 and 64 after tumor transplantation, whole-body GFP imaging was performed on the mice, under pentobarbital anesthesia, with the FluorVivo™ small animal imaging system (INDEC BioSystems, Capitola, CA, USA) in order to determine the extent of the primary tumor and metastasis (13).

Pathological evaluation of tumor status in liver. After the mice were sacrificed, the liver was fixed with IHC Zinc Fixative solution (BD Biosciences, San Diego, CA, USA). Tissue specimens were then prepared by conventional procedures and stained with H & E. Evaluation was carried out according to Shimosato *et al*. (14).

Oncogene expression in mesenteric lymph nodes. Expression of the human enhancer of the zeste homolog 2 (*hEZH2*) and human keratin 19 (*hKRT19*) genes in the mesenteric lymph nodes was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). EZH2 is a histone methyltransferase that regulates the survival and metastasis of cancer cells (15, 16), while KRT19 is a structural protein found in epidermal cells. We first confirmed expression of both genes in HCT-116 GFP cells. RNA was prepared in RNA-Later solution (Ambion, Austin, TX, USA). Table I shows the primers used in this experiment (15, 16). Mouse β-actin (mβ-actin) was used as the loading control for the tumor-transplanted mice, and human GAPDH (*hGAPDH*) for the HCT-116 cell lines. RT-PCR employed 35 cycles for *hEZH2* and *hKRT19*; 25 cycles for mβ-actin and *hGAPDH*.

Statistical analysis of data. Data were expressed as average values and standard deviations (SD). The Kaplan-Meier survival curve was analyzed by the log-rank and Wilcoxon tests, and the pathological results by an ANOVA, with a *p*-value of less than 0.05 being considered statistically significant.

Results

Efficacy of FOLFIRI on survival. Deaths due to tumor growth and metastasis began on day 40, and the number of deaths increased with time. The average survival time was 44.2±3.2 days in the control group; 48.6±1.4 days in the 5-FU + LV group; 66.3±3.9 days in the 5-FU + LV + CPT-11 (16 mg/kg) group; and 68.6±4.7 days in the 5-FU + LV + CPT-11 (24 mg/kg) group. Both the log-rank and Wilcoxon

Table I. *Primers used in this study.*

Gene		Primer sequence	Product size (bp)	Annealing temp. (˚C)	Cycles
Human *EZH2*	Sense	5'-TTGTTGGCGGAAGCGTGTAAAATC-3'	207	60.0	35
	Antisense	5'-TCCCTAGTCCCGCGCAATGAGC-3'			
Human *KRT19*	Sense	5'-TCGACAACGCCCGTCTG-3'	75	60.0	35
	Antisense	5'-CCACGCTCATGCGCAG-3'			
Human *GAPDH*	Sense	5'-GAAGGTGAAGGTCGGAGTC-3'	226	60.0	25
	Antisense	5'-GAAGATGGTGATGGGATTTC-3'			
Mouse *β-actin*	Sense	5'-ACCGTGAAAAGATGACCCAG-3'	528	53.0	25
	Antisense	5'-TACGGATGTCAACGTCACAC-3'			

Table II. *Efficacy of treatment on liver metastasis.*

35 days after transplantation	Control	5-FU + LV	FOLFIRI (CPT-11, 16 mg/kg)	FOLFIRI (CPT-11, 24 mg/kg)
Mice with metastasis/Total (%, positive rate)	5/7 (71%)	4/8 (50%)	1/9 (11%)	0/9 (0%)

tests demonstrated a statistically significant difference in survival time between the FOLFIRI groups and the control group, as well as 5-FU + LV group ($p<0.0001$) (Figure 1).

Slight weight loss or delay in weight gain was observed in some mice after completion of chemotherapy, but neither was significant, and weights later returned to within the normal range (data not shown).

Efficacy of FOLFIRI on metastasis. GFP imaging visualized the total area of the primary tumor and extent of metastasis. On day 35 after transplantation, the incidence of liver metastasis was greatly reduced in the FOLFIRI groups and eliminated in the FOLFIRI group using 24 mg/kg CPT-11 (Table II).

Pathological findings on the effect of FOLFIRI regimen in livers of transplanted mice. H&E staining confirmed that GFP imaging identified cancer metastasis in the liver (Figure 2).

Efficacy of FOLFIRI on expression of cancer genes in mesenteric lymph nodes of transplanted mice. The extent of *hEZH2* and *hKRT19* mRNA expression in mesenteric lymph nodes at autopsy were significantly lower in the FOLFIRI therapy groups than in the control group and the 5FU+LV group ($p<0.05$) (Figure 3, Table III).

Table III. *Expression of hEZH2 and hKRT19 in control and treated groups.*

Gene	Positive cases/Total (%)			
	Control	5-FU + LV	FOLFIRI (16 mg/kg)	FOLFIRI (24 mg/kg)
hEZH2	6/7 (86%)	6/6 (100%)	1/6 (17%)#,*	1/8 (13%)#,*
hKRT19	4/7 (57%)	5/6 (83%)	1/6 (17%)*	2/8 (25%)*

#Statistically significant at $p<0.05$ *versus* Control; *statistically significant at $p<0.05$ *versus* 5-FU + LV. Please see Materials and Methods for details.

Discussion

The results in this orthotopic model of colon cancer suggest that FOLFIRI prolongs survival through inhibition of metastasis, as well as of primary tumor growth.

Rajput *et al*., using GFP imaging of live BALB/c nude mice transplanted with GFP-HCT-116 onto the cecum, observed local proliferation of cancer cells by week 1 after transplantation and peritoneal dissemination and liver metastasis by week 4 (17). Histopathological analysis of autopsy tissue showed liver metastasis in 41% of the mice examined. In our study using NCR nude mice as hosts, histopathological analysis demonstrated liver metastasis in 100% (7/7) of the untreated mice. The HCT-116-GFP surgical orthotopic implantation (SOI) nude-mouse model is therefore a useful model of real-time monitoring of primary and metastatic tumor growth in living animals.

A number of studies have investigated the cytotoxic effects of FOLFIRI *in vitro* and *in vivo*. In an *in vitro* study, Inoue *et al*. reported that the cytotoxic effect of the combination of 5-FU and CPT-11 was sequence dependent, and that the optimal sequence of this combination therapy was to administer 5-FU followed by CPT-11 (18). Similarly, in an *in vivo* study, Cao and Rustum reported that the efficacy and adverse events (weight loss) of combination

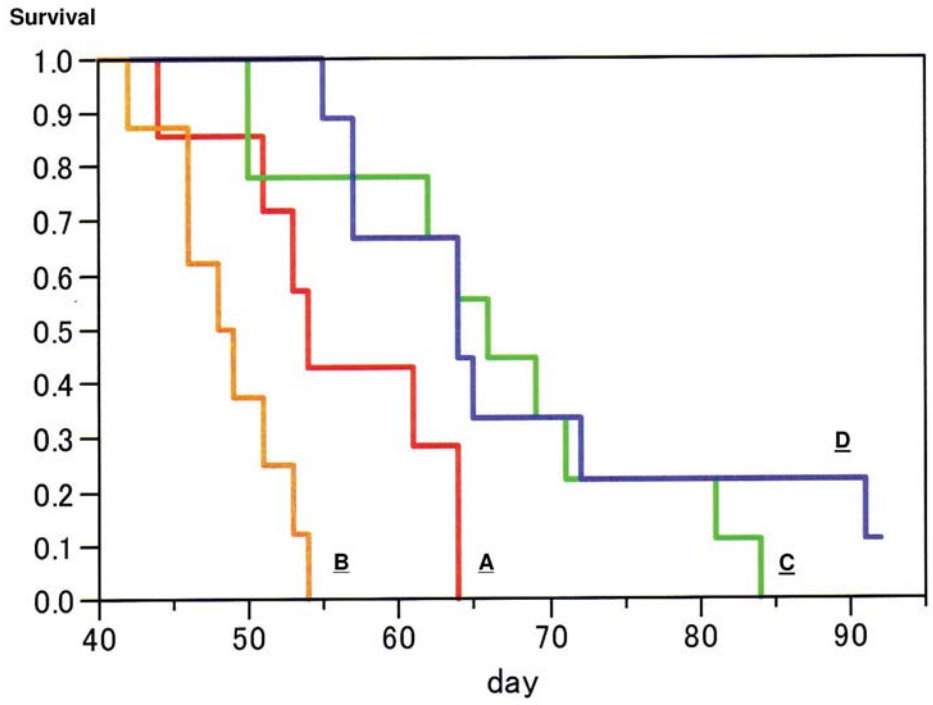

Figure 1. *Effect of treatment regimen on survival in mice transplanted orthotopically with GFP-HCT-116. a, Control group; b, 5-FU + LV group; c, 5-FU + LV + CPT-11 (16 mg/kg) group; d, 5-FU + LV + CPT11 (24 mg/kg) group, p<0.0001 (a vs. c, b vs. d).*

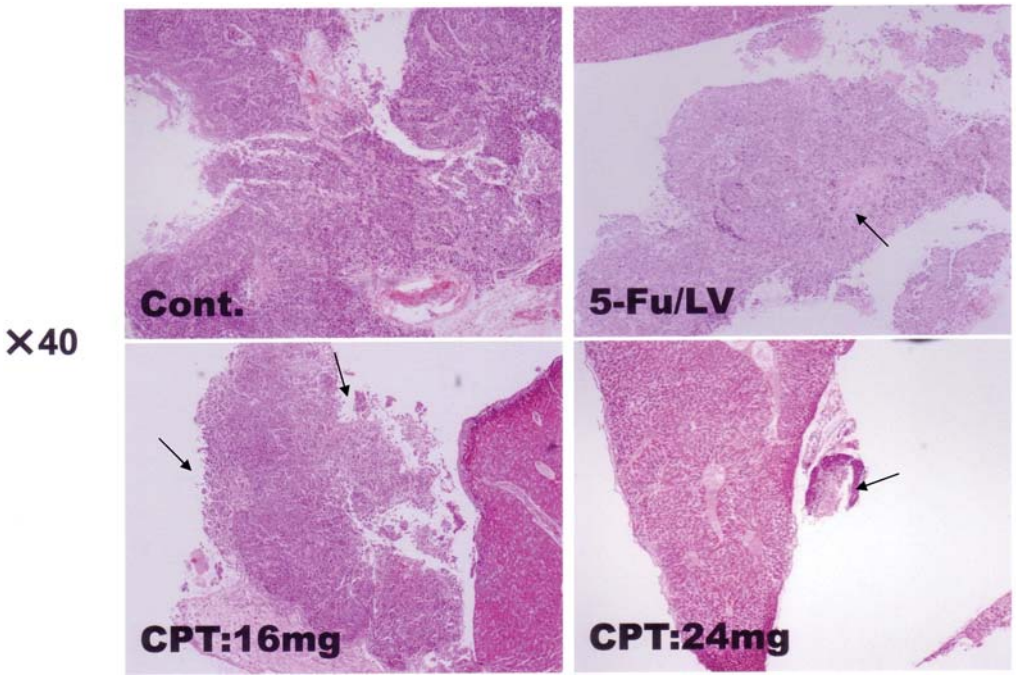

Figure 2. *Histological observation of liver in mice transplanted orthotopically with GFP-HCT-116 (H & E staining, ×40). Cont.: Control; 5-FU/LV: 5-FU + LV; 5-FU + LV + CPT-11 (16 mg/kg); 5-FU + LV + CPT-11 (24 mg/kg). Tumor in the liver of the control mouse was large and abundant in blood vessels (upper left micrograph). Tumor in mouse treated with 5-FU/LV was small, with necrosis in parts (arrow in upper right micrograph). Tumor in mouse treated with CTP-11 at 16 mg was smaller than in the former two (arrows in lower left micrograph). Tumor in mouse treated with CPT-11 at 24 mg/kg was extremely small (arrow in lower right micrograph) and the central area was almost necrotic.*

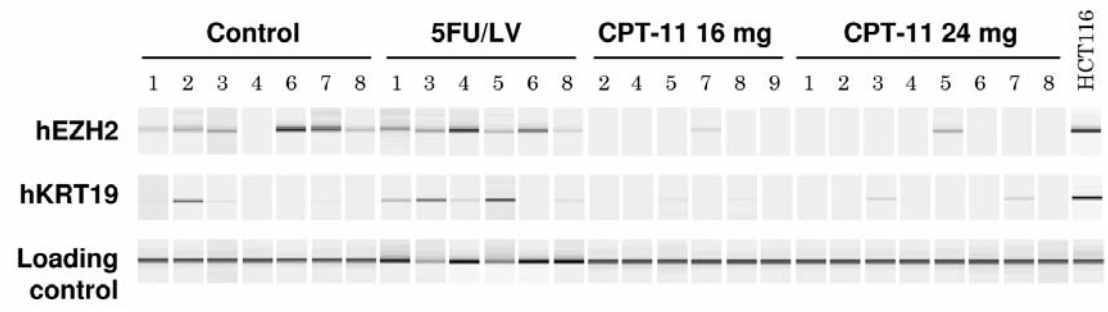

Figure 3. *Expression of cancer genes in mesenteric lymph nodes of mice transplanted orthotopically with human colon cancer line, GFP-HCT-116. See Materials and Methods for details.*

therapy with 5-FU and CPT-11 were sequence dependent in rats subcutaneously transplanted with colon cancer cells, and that reducing the dose of CPT-11 would negate adverse effects without reducing the anticancer efficacy of the regimen (19).

In the present study, we administered 5-FU at 30 mg/kg one hour after administration of LV at 50 mg/kg, followed 24 or 48 hours later by CPT-11 at either 16 mg/kg or 24 mg/kg. This cycle was repeated 3 times, with no acute adverse effects. We observed a clear inhibition of tumor growth and metastasis on day 35 after transplantation (13 days after completion of 3 cycles). Our results show that 5-FU administration followed by CPT-11 is effective.

Ishizu *et al*. compared different human colorectal cancer call lines in an experimental metastasis model with cells transplanted into the spleen of BALB/C *nu/nu* mice (20). They found HCT-116 to be the most metastatic, showing high fibronectin adhesion activity. CPT-11 significantly inhibited metastasis in this model, whereas 5-FU did not, exerting only a weak effect (20). Ji *et al*. found that CPT-11 administered to mice transplanted with HCT-116 onto the cecum significantly inhibited tumor proliferation and significantly reduced the length of tumor vessels (21).

Therefore, 5-FU and CPT-11 may have complementary activity in conferring the antitumor and antimetastatic efficacy of FOLFIRI. The results show the power of a clinically relevant orthotopic model and GFP imaging to demonstrate the antimetastatic efficacy of (candidate) drugs for metastasis, an urgent need in oncology (12, 22, 23).

Acknowledgements

The Authors would like to thank Assistant Professor A. Shibata, Department of Public Health, Kurume University, Professor P. Barron, International Medical Communication Center, Tokyo Medical University, and Professor J. Williams, Tokyo Dental College, Japan, for their advice on the data analysis and suggestions during the preparation of this article.

References

1 Marshall JL and Gehan EA: The development of novel agents for the treatment of colorectal cancer: a critical review of current practice and some suggestions for the future. Clin Adv Hematol Oncol *5*: 167-172, 2007.

2 Chung KY and Saltz LB: Adjuvant therapy of colon cancer: current status and future directions. Cancer J *13(3)*: 192-197, 2007.

3 Douilland JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L and Rougier P: Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomized trial. Lancet *355*: 1041-1047, 2000.

4 Tournigand C, Andre T, Achille E, Lledo G, Flesh M, Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, Louvet C and de Gramont A: FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. J Clin Oncol *22*: 229-237, 2004.

5 Noordhuis P, Holwerda U, Van der Wilt CL, Van Groeningen CJ, Smid K, Meijer S, Pinedo HM and Peters GJ: 5-Fluorouracil incorporation into RNA and DNA in relation to thymidylate synthase inhibition of human colorectal cancers. Ann Oncol *15*: 1025-1032, 2004.

6 Morgan RG: Leucovorin enhancement of the effects of the fluoropyrimidines on thymidylate synthase. Cancer *63(6 Suppl)*: 1008-1012, 1989.

7 Wallin A, Svanvik J, Holmlund B, Ferreud L and Sun XF: Anticancer effect of SN-38 on colon cancer cell lines with different metastatic potential. Oncol Rep *19(6)*: 1493-1498, 2008.

8 Tschmelitsch J, Barendswaard E, Williams C Jr, Yao TJ, Cohen AM, Old LJ and Welt S: Enhanced antitumor activity of combination radioimmunotherapy (131I-labeled monoclonal antibody A33) with chemotherapy (fluorouracil). Cancer Res *57*: 2181-2186, 1997.

9 Behr TM, Blumenthal RD, Memtsoudis S, Sharkey RM, Gratz S, Becker W and Goldenberg DM: Cure of metastatic human colonic cancer in mice with radiolabeled monoclonal antibody fragments. Clin Cancer Res *6*: 4900-4907, 2000.

10 Yamamoto N, Jiang P, Yang M, Xu M, Yamauchi K, Tsuchiya H, Tomita K, Wahl GM, Moossa AR and Hoffman RM: Cellular dynamics visualized in live cells *in vitro* and *in vivo* by differential dual-color nuclear-cytoplasmic fluorescent-protein expression. Cancer Res *64*: 4251-4256, 2004.

11 Yang M, Jiang P and Hoffman RM: Whole-body subcellular multicolor imaging of tumor-host interaction and drug response in real time. Cancer Res *67*: 5195-5200, 2007.
12 Hoffman RM: Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. Investigational New Drugs *17*: 343-359, 1999.
13 Yang M, Reynoso J, Bouvet M and Hoffman RM: A transgenic red fluorescent protein-expressing nude mouse for color-coded imaging of the tumor microenvironment. J Cell Biochem *106*: 279-284, 2009.
14 Shimosato Y, Oboshi S and Baba K: Histological evaluation of effects of radiotherapy and chemotherapy for carcinomas. Jpn J Clin Oncol *1*: 19-35, 1971.
15 Kurahashi T, Hara I, Oka N, Kamidono S, Eto H, and Miyake H: Detection of micrometastases in pelvic lymph nodes in patients undergoing radical cystectomy for locally invasive bladder cancer by real-time reverse transcriptase-PCR for cytokeratin 19 and uroplakin II. Clin Cancer Res *11*: 3773-3777, 2005.
16 Sudo T, Utsunomiya T, Mimori K, Nagahara H, Ogawa K, Inoue H, Wakiyama S, Fujita H, Shirouzu K and Mori M: Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. Br J Cancer *92*: 1754-1758, 2005.
17 Rajput A, Dominguez San Martin I, Rose R, Beko A, Levea C, Sharratt E, Mazurchuk R, Hoffman RM, Brattain MG and Wang J: Characterization of HCT116 human colon cancer cells in an orthotopic model. J Surg Res *147*: 276-281, 2008.
18 Inoue Y, Tanaka K, Hiro J, Yoshiyama S, Toiyama Y, Eguchi T, Miki C and Kusunoki M: *In vitro* synergistic antitumor activity of a combination of 5-fluorouracil and irinotecan in human colon cancer. Int J Oncol *28*: 479-486, 2006.
19 Cao S and Rustum YM: Synergistic antitumor activity of irinotecan in combination with 5-fluorouracil in rats bearing advanced colorectal cancer: role of drug sequence and dose. Cancer Res *60*: 3717-3721, 2000.
20 Ishizu K, Sunose N, Yamazaki K, Tsuruo T, Sadahiro S, Makuuchi H and Yamori T: Development and characterization of a model of liver metastasis using human colon cancer HCT-116 cells. Biol Pharm Bull *30*: 1779-1783, 2007.
21 Ji Y, Hayashi K, Amoh Y, Tsuji K, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, Bouvet M and Hoffman RM: The camptothecin derivative CPT-11 inhibits angiogenesis in a dual-color imageable orthotopic metastatic nude mouse model of human colon cancer. Anticancer Res *27*: 713-718, 2007.
22 Hoffman RM: The multiple uses of fluorescent proteins to visualize cancer *in vivo*. Nature Rev Cancer *5*: 796-806, 2005.
23 Steeg PS, Anderson RL, Bar-Eli M, Chambers AF, Eccles SA, Hunter K, Itoh K, Kang Y, Matrisian LM, Sleeman JP, Theodorescu D, Thompson EW and Welch DR: Preclinical drug development must consider the impact on metastasis. Clin Cancer Res *15*: 4529-4530, 2009.

Received September 3, 2009
Revised January 21, 2010
Accepted January 22, 2010