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# Antimetastatic Efficacy of Oral 5-FU Imaged by Green Fluorescent Protein in Real Time

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**Abstract.** *The effectiveness of oral 5-FU in suppressing liver metastasis was assessed in a highly-metastatic mouse model. Doses of 20 and 25 mg/kg oral 5-FU significantly suppressed primary and metastatic tumor growth ( $p=0.012$ ). These inhibitory effects were more dramatic in suppressing liver metastasis ( $p=0.0$ ). The efficacy of 5-FU was visualized by whole-body fluorescence imaging of the green fluorescent protein-expressing tumor and its subsequent metastases. Toxicity was observed only in the 30 mg/kg dose. Furthermore, we showed that the non-toxic doses of 5-FU significantly prolonged survival in these animals. These data suggest the important clinical potential of oral 5-FU.*

Fluorouracil (5-FU), a pyrimidine analog, is used for gastrointestinal and other cancers (2-5). Unfortunately, 5-FU has had limited efficacy (6-9). However, the efficacy of 5-FU can be enhanced if it is administered with agents such as leucovorin or by regional administration to the liver and peritoneal cavity (5,8).

5-FU is converted to two activated forms: 5-fluorouridine triphosphate (FUTP) and 5-fluoro-2'-deoxyuridine monophosphate (FdUMP) (15-19). FUTP and FdUMP act at both RNA and DNA levels (15-19). In order to improve 5-FU efficacy, we investigated the effects of oral 5-FU on liver metastasis in a highly metastatic fluorescent mouse model.

Strong fluorescent labeling with green fluorescent protein (GFP) along with inexpensive video detectors, positioned external to the mouse, allowed the monitoring of details of tumor growth and metastatic spread in the mouse model (20-23).

## Materials and Methods

*Animals.* Male athymic NCR nude mice between 5 and 6 weeks of age were used in this study. The animals were bred and maintained in a

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**Key Words:** 5-Fluorouracil, antimetastatic effects, green fluorescent protein whole-body imaging.

HEPA-filtered environment with cages, food and bedding sterilized by autoclaving. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

*Green fluorescent protein (GFP) expression vector (20-23).* The retroXpress vector pLEIN was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The pLEIN vector expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message. pLEIN was produced in PT67 packaging cells.

For vector production, PT67 cells, at 70% confluency, were incubated with a precipitated mixture of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl-sulfate reagent and saturating amounts of pLEIN plasmid for 18hours. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 hours post transfection. For selection, the cells were cultured in the presence of 200-1000  $\mu\text{g/ml}$  G418 for 7days.

*In vivo GFP transduction.* For *in vivo* GFP gene transduction, two fragments (1  $\text{mm}^3$ ) of the AC3488 liver metastatic model, derived from a liver metastases of a patient with colon cancer, were implanted in the colon of each of 3 nude mice as described below. Twenty-four hours after the tumor fragments were implanted, 1 ml of retroviral supernatant of PT 67 cells was injected into the peritoneal cavity of the nude mice once every day for 5 days. The nude mice were sacrificed to harvest the GFP-expressing tumor fragments two weeks after injection of retroviral supernatants of PT 67 cells. The selected GFP-expressing fragments were re-transplanted in the colon of additional mice for further selection of GFP expression.

*Metastatic animal model.* A tumor fragment (1  $\text{mm}^3$ ) from a GFP-expressing AC3488 liver metastasis (24,25) from a single animal was implanted as follows: after proper exposure of the colon following a lower midline abdominal incision, the serosa of the colon was removed and two pieces of 1  $\text{mm}^3$  tumor fragment per mouse were implanted. An 8-0 surgical suture was used to penetrate these small tumor pieces and suture them on the wall of the intestine. The intestine was then returned to the abdominal cavity. The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer. The animals were kept under isoflurane anesthesia during surgery. All procedures of the operation described above were performed with a 7  $\times$  magnification microscope (Olympus). The animals were kept in a barrier facility under HEPA filtration.

*Whole-body optical imaging of GFP-expressing tumor growth and metastasis (20-23).* Microscopy A Leica fluorescence stereo microscope model LZ12 equipped with a mercury 50W lamp power supply was used. To visualize GFP and fluorescence, excitation was produced through a

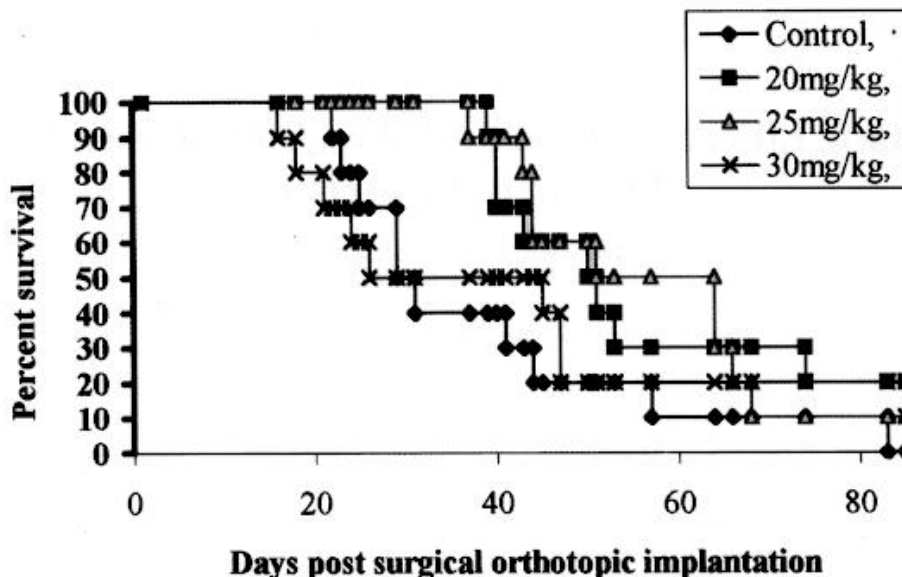


Figure 1. Percent survival rate is compared for the different doses of 5-FU and the control group. Twenty and 25 mg/kg doses of 5-FU increased survival compared to the control. 5-FU at 30 mg/kg was toxic. Unlike the 5-FU-treated mice, none of the animals in the control group survived the duration of the study.

D425/60 band pass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long pass filter GG475 (Chroma Technology, Brattleboro, VT, USA). Macroimaging was carried out in a light box (Lighttools Research, Encinitas, CA, USA). Fluorescence excitation of GFP tumors was produced through an interference filter 440+/-20 nm using slit fiber optics for animal illumination. Fluorescence was observed through a 520 nm long pass filter. Images from the microscope and light box were captured on a Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems, Bridgewater, NJ, USA). Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 3.1 software (Media Cybernetics, Silver Springs, MD, USA). High-resolution images of 1,024 x 724 pixels were captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR, model SLV-R1000 (Sony, Tokyo, Japan).

**Analysis of metastases.** The time course of tumor progression was tracked by GFP fluorescence whole-body imaging after transplantation. Periodically, the tumor-bearing mice were examined in the fluorescence light box. The time of tumor occurrence in different organs and the numbers of metastases were recorded.

**Study design.** Three days after implantation, the mice were randomized into 4 different groups of 10 mice each for treatment purposes. Groups 1-3 received 20, 25, and 30 mg/kg of 5-FU, respectively. Group 4 served as the negative control and did not receive any treatment. 5-FU (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) was diluted in water and administered orally. Dosing was performed everyday for three weeks. GFP imaging was performed on each mouse twice a week.

## Results and Discussion

The survival time of the mice in each of the treated groups was compared to that of the vehicle by the log rank test. The

mice in the 20 and 25 mg/kg 5-FU groups had a mean survival time of approximately 56 days for both doses compared to 40 days for control ( $p=0.042$  and  $0.02$ , respectively). As can be seen in Figure 1, 30% of the animals in the 30 mg/kg group died between days 13 and 19 of the study indicating toxicity. None of the animals in the other three groups died during this period of time (Figure 1). Figure 1 shows the extended survival of the 20 and 25 mg/kg groups.

A series of whole-body GFP images show the real-time progression of primary and metastatic tumor growth in the control and the 20, 25/ and 30mg/kg 5-FU groups (Figures 2-4). These whole-body images over the abdominal area showed that the primary and the metastatic tumor areas increased less in the treated groups than the control groups (Figures 2-4) ( $p=0.012$ ) for both the 20 and 25 mg/kg groups.

Transforming the tumors with GFP enables better detection of primary tumor growth and metastasis (20-23). Visualizing cancer cells that stably express GFP *in vivo* is far more sensitive and rapid than the traditional cumbersome procedures of histopathological examination or immunohistochemistry, which also require sacrifice of the animal. GFP expression in the tumor cells is stable over indefinite time periods, allowing the quantitative imaging of tumor growth and metastasis formation. The very bright GFP fluorescence enables internal tumors and metastases to be externally observed in critical organs such as the liver. No contrast agents, substrates, radioactive materials, anesthesia, or treatment need to be administered to the animals; just blue light illumination is necessary (20-23).

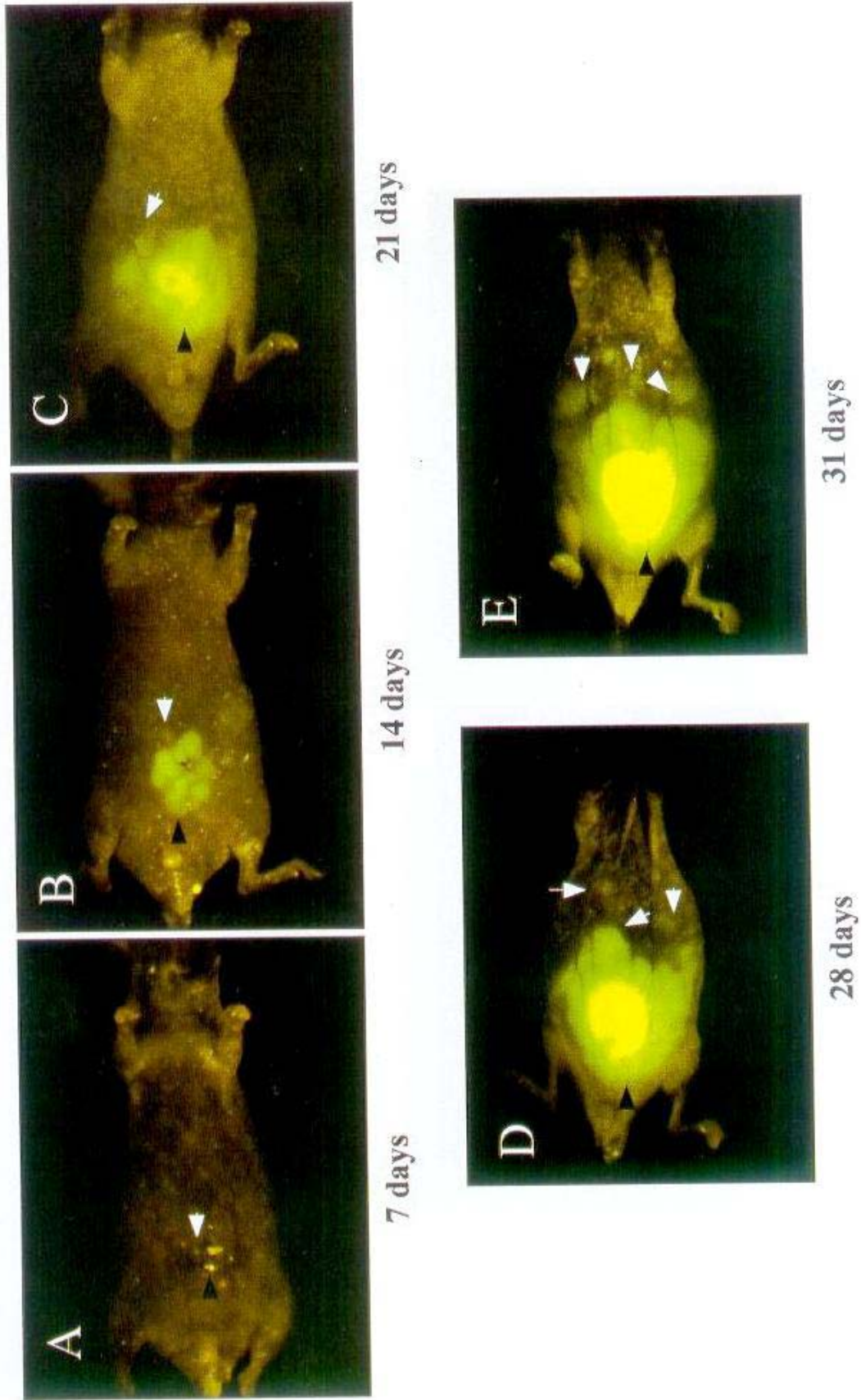


Figure 2. Real-time whole-body images of AC3488-GFP-tumor progression. A series of external fluorescence images visualizes primary (black arrows) and liver metastatic growth (white arrow) in a single mouse in the control group. Images were obtained from day 7 to day 31 post surgical orthotopic implantation (SOI).

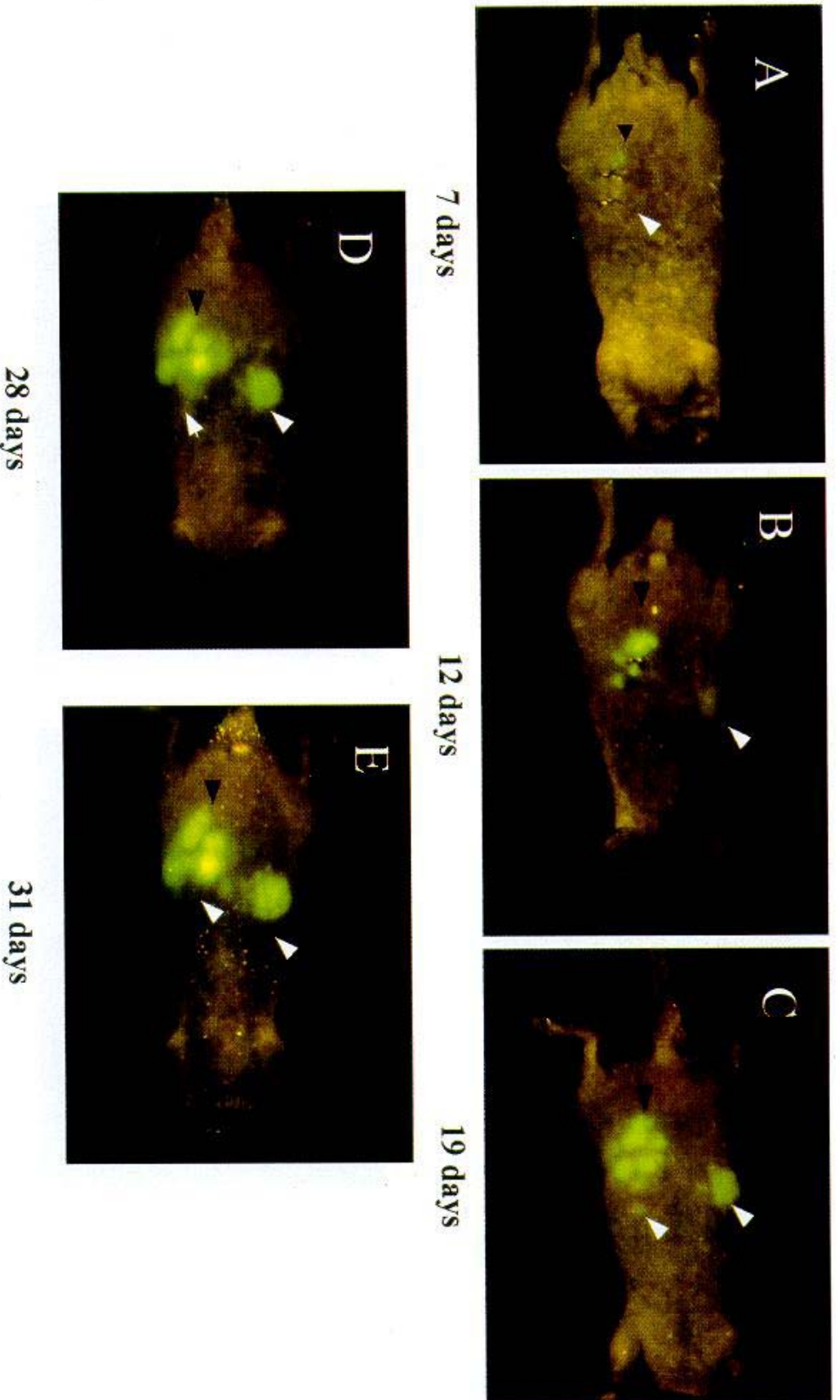


Figure 3. Real-time whole-body images of ACC3488-GFP-tumor expression. A series of external fluorescence images visualizes primary (black arrow) and liver metastatic growth (white arrow) in a single mouse treated with 20 mg/kg 5-FU. Images were obtained from day 7 to day 31 post surgical orthotopic implantation (SOI). The primary and metastatic areas are smaller compared to untreated animals (refer to Figure 2).

Three different doses of 5-FU (20, 25 and 30 mg/kg) were compared against control mice that received no treatment. The results showed that 5-FU prolonged the survival time of the treated animals at doses of 20 mg/kg and 25 mg/kg (Figure 1). Statistically significant differences in survival were observed between these two groups and the control ( $p=0.042$  for 20 mg/kg of 5-FU and  $p=0.02$  for 25 mg/kg of 5-FU). 5-FU was toxic at 30 mg/kg. Both primary and liver metastasis tumor areas were significantly smaller, as measured by GFP whole-body imaging for all doses of 5-FU, compared to the control mice (Figures 2-4). These data suggest that oral doses of 20-25 mg/kg 5-FU result in suppression of metastatic tumor growth. These data suggest that clinical efficacy may also be achieved with preventing metastasis using oral 5-FU.

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Received August 29, 2002  
Accepted September 30, 2002