

# A Photon Counting Technique for Quantitatively Evaluating Progression of Peritoneal Tumor Dissemination

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## Abstract

We recently established a mouse model of peritoneal dissemination of human gastric carcinoma, including the formation of ascites, by orthotopic transplantation of cultured gastric carcinoma cells. To clarify the processes of expansion of the tumors in this model, nude mice were sacrificed and autopsied at different points of time after the orthotopic transplantation of the cancer cells for macroscopic and histopathologic examination of the tumors. The cancer cells grew actively in the gastric submucosa and invaded the deeper layers to reach the serosal plane. The tumor cells then underwent exfoliation and became free followed by the formation of metastatic lesions initially in the greater omentum and subsequent colonization and proliferation of the tumors on the peritoneum. Although this model allowed the detection of even minute metastases, it was not satisfactory from the viewpoint of quantitative and objective evaluation. To resolve these problems, we introduced a luciferase gene into this tumor cell line with a high metastasizing potential and carried out *in vivo* photon counting analysis. This photon counting technique was found to allow objective and quantitative evaluation of the progression of peritoneal dissemination on a real-time basis. This animal metastatic model is useful for monitoring the responses of tumors to anticancer agents. (Cancer Res 2006; 66(15): 7532-9)

## Introduction

Tumor dissemination and ascites are the two major features of cancerous peritonitis. Of the various manifestations of the progression of cancer affecting the i.p. organs (gastric, hepatic, ovarian, and other cancers), cancerous peritonitis is the most closely associated with poor operative results (1–6). In particular, scirrhous gastric cancer (diffusely infiltrative carcinoma or Borrmann's type IV carcinoma or the linitis plastica type) is a high-grade gastric cancer that is difficult to detect in the early stages and is often complicated by peritoneal dissemination (7–9). Although peritoneal dissemination is an important subject, very few experimental studies have been conducted to characterize its occurrence. In general, most of the experimental models of peritoneal dissemination from gastric cancer established to date have involved direct i.p. implantation of cancer cells (10–12). Although these conventional models may allow limited examina-

tion of the later stages of peritoneal dissemination, they cannot be expected to allow reasonable evaluation of its early stages. It is well known that implanting human tumor fragments and tumor cells orthotopically into the corresponding organs of nude mice results in much higher metastatic rates (13, 14). However, only one orthotopic implantation model, scirrhous carcinoma of the stomach, has been reported (15). We recently established two scirrhous gastric carcinoma-derived tumor cell lines capable of spontaneous metastasis following ectopic implantation (16). We repeated cycles of orthotopic transplantation of these tumor cell lines, collected cancer cells from the ascitic fluid formed as a result of cancerous peritonitis, and used the collected cells for further cycles of orthotopic transplantation. In this way, we isolated cell lines (44As3, 58As1, and 58As9) with high metastasizing potential and stable metastatic characteristics (17). When these cells were implanted orthotopically into the animals, bloody ascites formed within 3 to 5 weeks, resulting in the death of the animals.

As stated above, conventionally, progression of peritoneal dissemination has been analyzed by implanting cancer cells directly into the peritoneal cavity followed by sacrifice and autopsy of the animals at certain points of time after implantation and, finally, measurement of the number and weight of the tumor nodules in the sacrificed animals (18–20). Evaluation of the efficacy of anticancer agents was also hampered by this limitation (21–25). Evaluation using these methods may be affected by subjective factors and, therefore, unsatisfactory from the viewpoint of quantitative or objective evaluation. In order for our animal model of peritoneal dissemination to be applied universally as a drug evaluation system, we needed to establish a method for quantitative observation and objective evaluation of the relevant variables.

Recent progress in the optical imaging of cancers in animal models presents many potential advantages for recreating the disease process, disease detection, screening, diagnosis, drug development, and treatment evaluation. Fluorescence-based imaging (26–35) and photon counting analysis (36–43) modalities are well developed and allow specific, highly sensitive and quantitative measurements of a wide range of tumor-related variables in mice. Herein, we have shown that photon counting technique is an effective technology in living mice.

## Materials and Methods

**Established highly metastatic cell lines and culture.** 44As3, highly peritoneal metastatic cell line, and parent HSC-44PE, human scirrhous gastric carcinoma-derived cell line, were previously reported (16, 17). When the subclones isolated by repeated s.c. injection of HSC-44PE cells were implanted orthotopically, they spread to the greater omentum, mesenterium, etc. and caused the formation of bloody ascites in a few animals (16). We repeated cycles of isolation of ascitic tumor cells and orthotopic inoculation of these cells, in turn, into animals to isolate highly metastatic

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44As3 cell lines, having a strong capability of inducing the formation of ascites (17).

The cell lines were maintained in RPMI 1640 supplemented with 10% FCS (Sigma Chemical, St. Louis, MO), 100 IU/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate (Immuno-Biological Laboratories, Takasaki, Japan) in a 5% CO<sub>2</sub> and 95% air atmosphere at 37°C (17).

**In vivo photon counting analysis.** 44As3 and HSC-44PE cells were transfected with a complex of 4 µg pEGF-PLuc plasmid DNA (Clontech, Palo Alto, CA) and 24 µL GeneJammer reagent (Stratagene, Cloning Systems, La Jolla, CA) in accordance with the manufacturer's instructions. Stable transfectants were selected in geneticin (400 µg/mL; Invitrogen, Carlsbad, CA), and bioluminescence was used to screen transfected clones for luciferase gene expression using the IVIS system (Xenogen, Alameda, CA). Clones expressing the luciferase gene were named 44As3Luc and HSC44Luc.

Orthotopic implantation of  $1 \times 10^6$  44As3Luc and HSC44Luc cells was conducted in 6-week-old female BALB/c-*nu/nu* mice (day 0) as described previously (17). *In vivo* photon counting analysis was conducted on a cryogenically cooled IVIS system using Living Image acquisition and analysis software (Xenogen) as described previously (39).

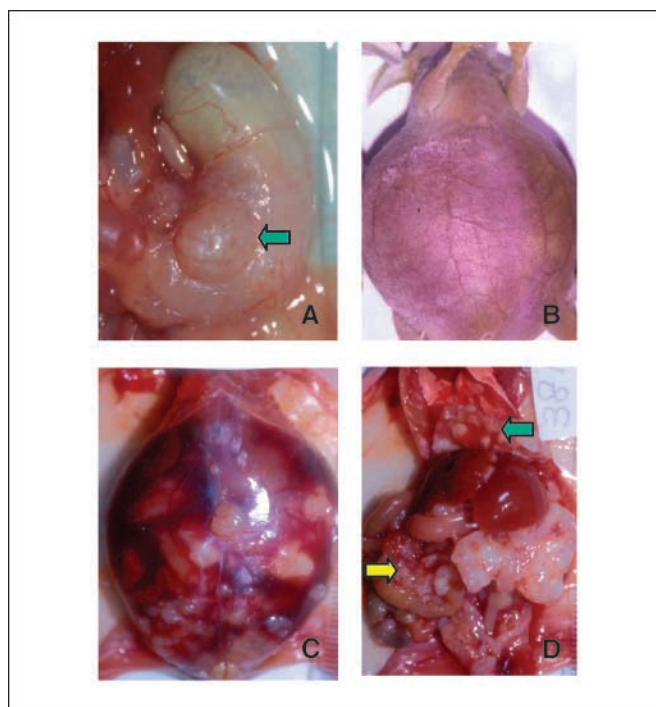
Animal protocols were approved by the committee for Ethics of Animal Experimentation and were in accordance with the Guideline for Animal Experiments in the National Cancer Center. Mice were purchased from CLEA Japan (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions and provided with sterile food, water, and cages. Ambient light was controlled to provide regular cycles of 12 hours of light and 12 hours of darkness.

**Therapeutic study with irinotecan (CPT-11).** The experimental mice were divided into a control group that received vehicle alone (saline) and experimental groups that received i.v. inoculation of 200 mg/kg/mouse of CPT-11, a clinically active topoisomerase I inhibitor, a level that has been reported to be highly effective in tumor growth (17). On days 3, 7, and 11, tumor-bearing mice received an i.v. injection of CPT-11. The additional injection of CPT-11 was done on days 28, 31, and 35. CPT-11 was purchased from Yakult Honsha (Tokyo, Japan) and dissolved in saline before being injected.

**Statistical analysis.** All data were analyzed by using the unpaired *t* test and expressed as the mean  $\pm$  SE. A *P* < 0.05 was considered statistically significant.

## Results

**Animal model of peritoneal dissemination.** The highly metastatic peritoneal cell line used in this study (44As3) was isolated by repeated cycles of orthotopic implantation of HSC-44PE cells and collection of the ascitic tumor cells as described in Materials and Methods (16, 17). As shown in Table 1 and Fig. 1, the tumor formed by this cell line was characterized by a propensity



**Figure 1.** Macroscopic appearance of the peritoneal disseminations after orthotopic implantation of 44As3 cells. *A*, green arrow, orthotopic implantation of the cells in the stomach of nude mice was followed by tumor formation 3 weeks later. *B* and *C*, carcinomatous peritonitis was observed 5 weeks after orthotopic implantation of the cells. Abdominal distension because of bloody ascites was evident. *D*, peritoneal dissemination was recognized from the innumerable whitish nodules visualized in the abdominal cavity, mesenterium (yellow arrow), omentum, parietal peritoneum, and diaphragm (green arrow).

for early peritoneal dissemination and was frequently associated with the formation of ascites and the animals became moribund ~35 days after implantation. On the other hand, the graft cell survival after implantation of the parent cell line (HSC-44PE) was 67% and moribund animals were not seen until ~90 days after implantation, although no ascites formation was observed.

**Anatomic, histopathologic, and ultrastructural analysis of the progression of peritoneal dissemination.** To analyze the process of progression of peritoneal dissemination, 44As3 cells ( $1 \times 10^6$ ) were implanted orthotopically into the gastric wall of nude mice. Every 7 days after transplantation, five animals were

**Table 1.** Comparison of the survival and metastatic behavior of animals following orthotopic implantation of the highly metastatic and the parent cell lines

Cell line	Survival days	Tumor formation*	Ascites <sup>†</sup>	Disseminated metastasis				Lymph node	Liver	Pancreas <sup>‡</sup>	Kidney <sup>‡</sup>
				Omentum	Mesenterium	Peritoneum	Diaphragm				
44As3	35 $\pm$ 15 (22-65)	15/15 (100%)	14/15 (93%)	15/15	15/15	15/15	9/15	15/15	10/15	6/15	1/15
HSC-44PE	135 $\pm$ 48 (90-200)	10/15 (67%)	0/10 (0%)	5/10	3/10	3/10	0/10	5/10	0/10	0/10	0/10

\*Mice were sacrificed 200 days after the orthotopic implantation. Data are the number of mice bearing metastases at the site/total number of mice bearing tumor.

<sup>†</sup>Ascites formation: >0.5 mL of ascitic fluid.

<sup>‡</sup>Micrometastases.

**Table 2.** Detection of metastasis and peritoneal dissemination after the orthotopic implantation of 44As3 cells

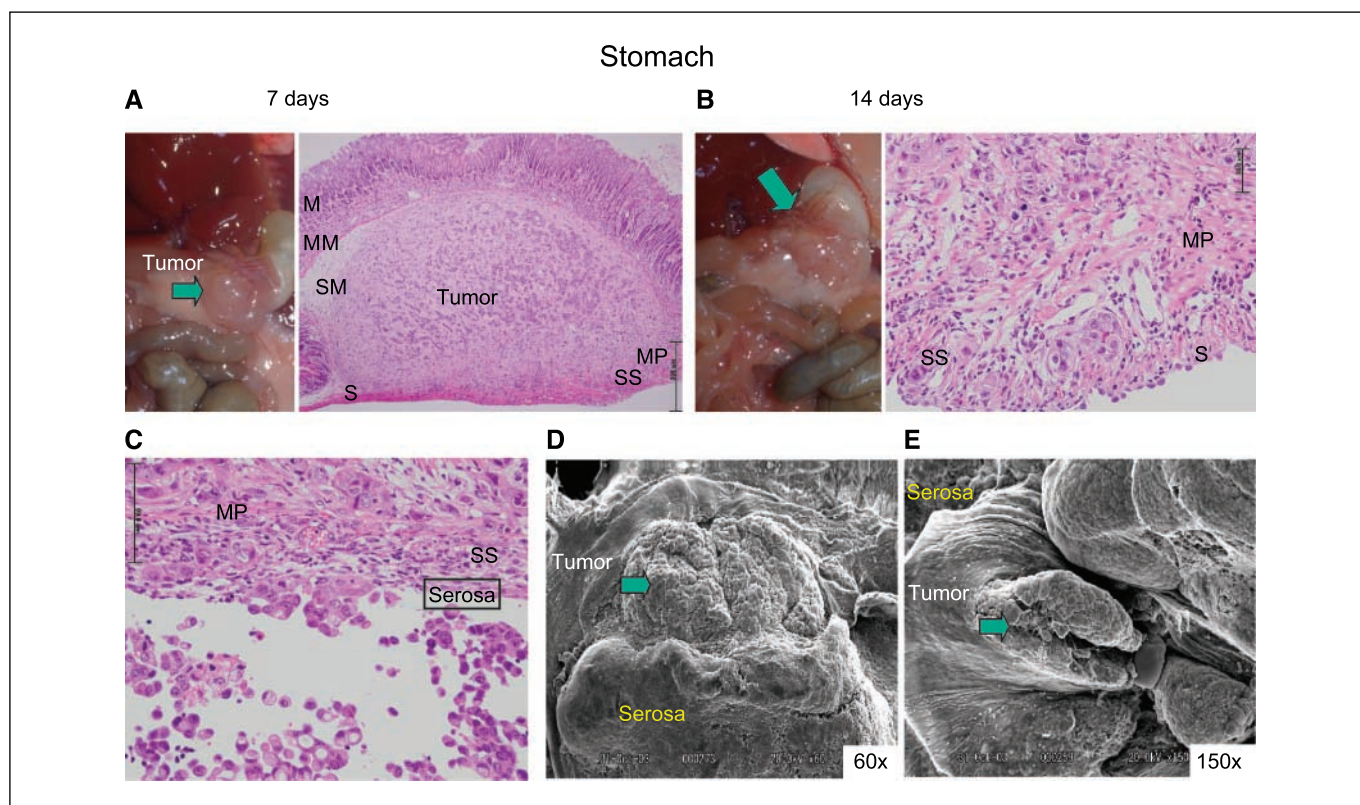
Days	Stomach	Ascites*	Disseminated metastasis				Lymph node	Liver	Pancreas <sup>†</sup>	Kidney <sup>†</sup>
			Omentum	Mesenterium	Peritoneum	Diaphragm				
7	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
14	5/5	0/5	3/5	0/5	0/5	0/5	1/5 <sup>†</sup>	0/5	1/5	0/5
21	5/5	1/5	5/5	3/5	3/5	0/5	2/5	1/5	1/5	0/5
28	5/5	3/5	5/5	5/5	5/5	2/5	5/5	1/5	2/5	0/5
35	5/5	5/5	5/5	5/5	5/5	3/5	5/5	2/5	2/5	1/5

\*Ascites formation: &gt;0.5 mL of ascitic fluid.

†Micrometastases.

sacrificed and subjected to postmortem examination for macroscopic, histopathologic, and ultrastructural analyses (Table 2; Fig. 2). The metastatic cells (44As3) proliferated actively in the submucous tissue of the stomach (Fig. 2A) and began to infiltrate in the lymphatics on the 7th day. During the 2nd week following transplantation, the tumor grew more rapidly within the gastric wall, with invasion of the muscularis propria and the subserosal tissue (Fig. 2B). In some mice showing rapid growth of the tumor, the cancer cells broke through the serosa to become exfoliated and freed (Fig. 2C). These exfoliated and freed cancer cells could be

visualized under the scanning electron microscope (Fig. 2D and E). Peritoneal dissemination began to be noted in the 2nd week, with cells on the greater omentum (Table 2). Micrometastases to the lymph nodes and pancreas were also noted, although not frequently. By the 3rd week, the foci of metastasis were noted in the greater omentum, mesenterium, and peritoneum. Scanning electron microscopy revealed the proliferation of the cancer cells (e.g., those colonizing the mesenterium) with the formation of larger cell clusters (data not shown). In the peritoneum, colonization of the freed cancer cells and their interaction with



**Figure 2.** Macroscopic and microscopic appearance of the tumor growth of stomach of nude mice after orthotopic implantation of 44As3 cells. *A*, green arrow, orthotopic implantation of 44As3 cells in the stomach of nude mice was followed by tumor formation 7 days later. Actively proliferating 44As3 cells in the gastric submucosa (H&E). *M*, mucosa; *MM*, muscularis mucosae; *SM*, submucosa; *MP*, muscularis propria; *SS*, subserosa; *S*, serosa. *B*, tumor invasion of the muscularis propria and subserosal tissue (H&E). *C*, note 44As3 cells breaking through the serosa and becoming exfoliated and free (H&E). *D* and *E*, visualization of cancer cells breaking through the serosa and becoming exfoliated and free. Mice were sacrificed, and the tissues were examined for metastasis in various organs and processed for histologic examination as described (47, 48). Scanning electron microscopic examination was done according to standard procedures (49).

mesothelial cells were visualized (data not shown). By the 4th week, metastases to the greater omentum, mesenterium, peritoneum, and lymph nodes were noted and some animals also showed additional metastasis to the diaphragm (Table 2). Metastasis to the liver was occasionally seen. In some mice, in which the tumors grew rapidly, formation of ascites began to be noted ~21 days after the orthotopic implantation. Some of these animals became moribund on the 28th day (Tables 1 and 2). By the 35th day, all the animals showed metastasis, with dissemination to the greater omentum, mesenterium, and peritoneum accompanied by the formation of bloody ascites as well as lymph node metastasis (Table 2). Metastasis to the diaphragm was also seen frequently. Micrometastasis to the kidneys was noted in a few animals.

**Analysis of the progression of dissemination using luciferase gene-transfected cells.** The analytic method described above allows detailed evaluation even of micrometastases. However, it has limitations from the viewpoint of quantitative and objective analysis. To resolve these problems, we introduced the luciferase gene into the metastatic 44As3 cell line and its parent cell line HSC-44PE by means of liposome transfer; this yielded cells with high luciferase activity, 44As3Luc and HSC44Luc, respectively. When the 44As3Luc cells ( $1 \times 10^6/100 \mu\text{L}$ ) were implanted s.c. into nude mice, a significant correlation was observed between tumor growth (volume) and the luciferase emission level (photon number; Fig. 3). Both cell lines were therefore used for the subsequent experiments.

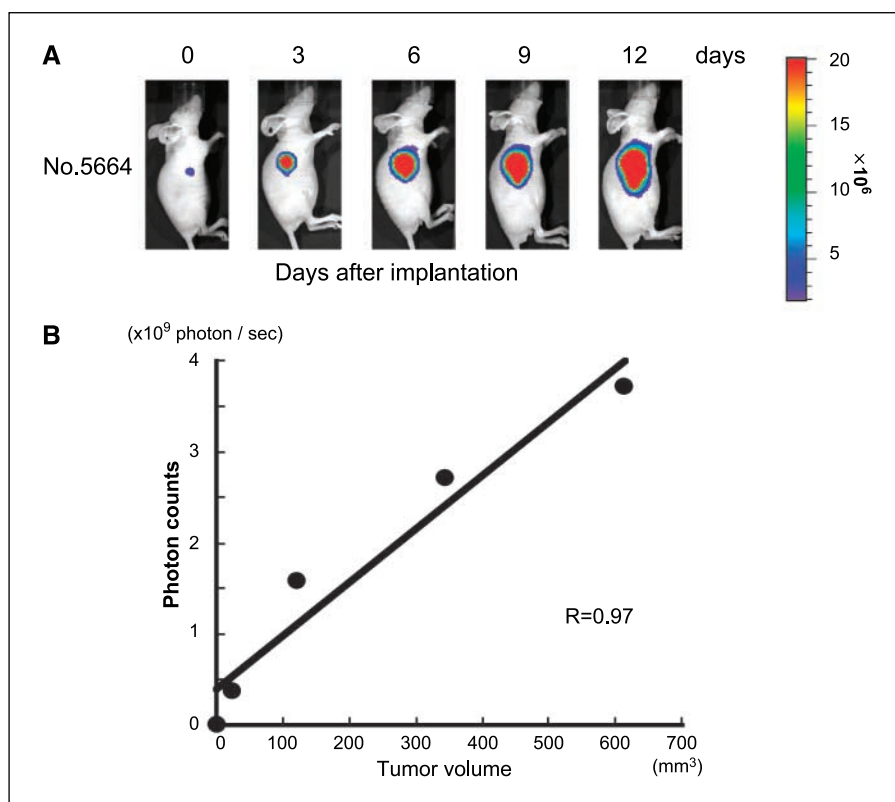
The metastatic 44As3Luc or its parent cell line HSC44Luc cells were implanted orthotopically into nude mice. With the light emission noted at the site of implantation, photon counting analysis was thereafter carried out at intervals of 3 or 4 days. Figure 4A (top) presents a typical example. Chronological observation of the same animals, which were kept alive, was possible by this method. The 44As3Luc cells proliferated actively in the

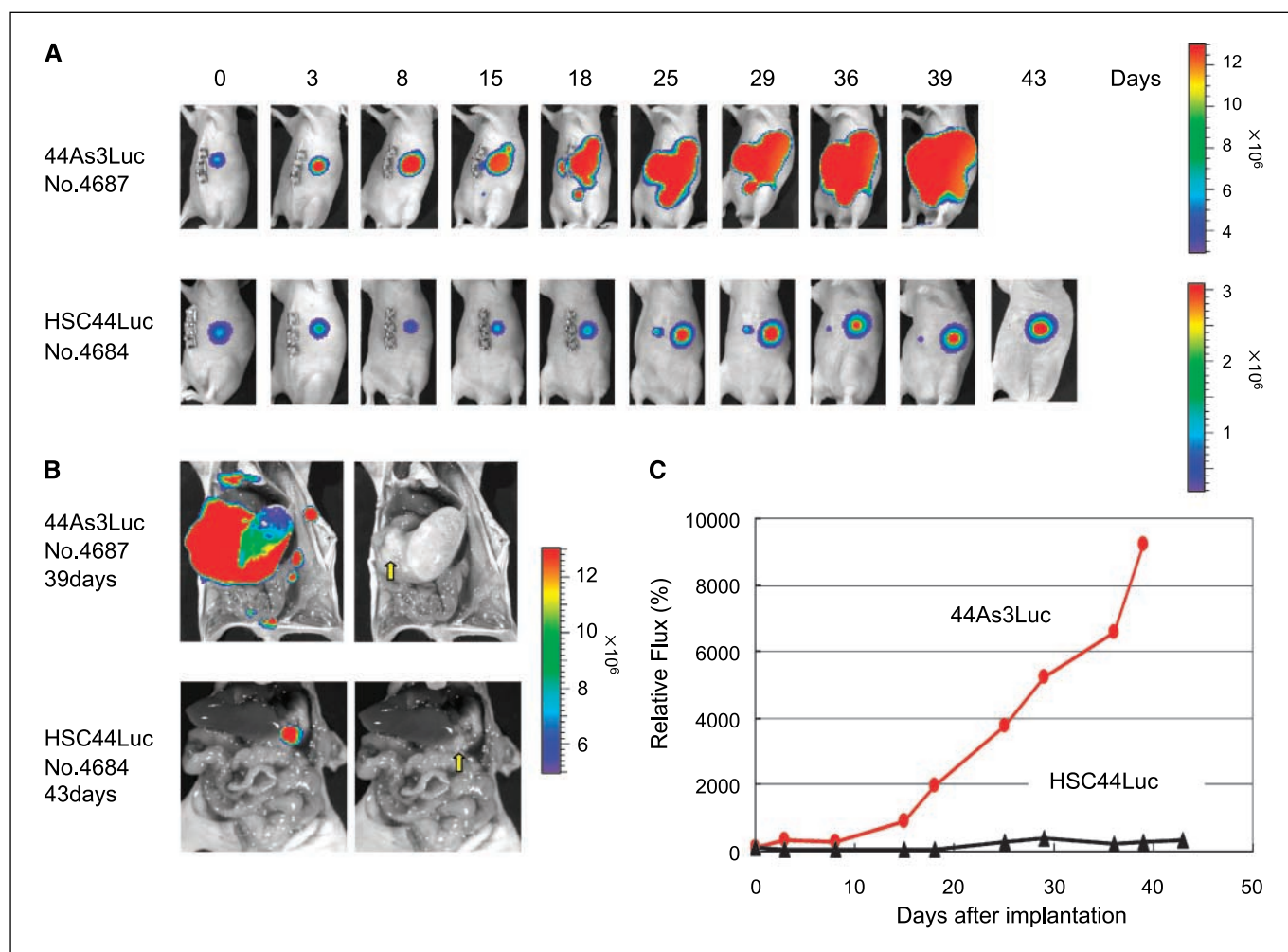
stomach. By the 15th day after implantation, tumor invasion of the peritoneal cavity and gradual progression of dissemination and increases in the sizes of the cell clusters were observed. Around the 25th day after implantation, a marked increase in the volume of the ascitic pool was noted by macroscopic observation, and some moribund mice were observed after the 29th day. When the moribund animals were sacrificed for autopsy, dissemination to the mesenterium and parietal peritoneum was often observed, frequently accompanied by metastasis to the lymph nodes. It was confirmed anatomically and histopathologically that the light-emitting sites corresponded to the tumor-affected sites (Fig. 4B). On the other hand, in the animals transplanted with the HSC44Luc, the tumor growth tended to be confined to the region of the stomach where the cells had been implanted (Fig. 4B), with slower tumor cell proliferation. As shown in Fig. 4A (bottom), luminescence was sometimes noted in the lymph nodes around the stomach and so on, but all of these foci of metastasis had regressed by ~60 days after implantation. Moribund animals began to be observed by the 85th day, although no ascites formation was noted in any of the animals.

By plotting the number of photons against time, a tumor growth curve reflecting the progression of peritoneal dissemination was obtained. When the relative number of photons from the highly metastatic cell line 44As3Luc and its parent cell line HSC44Luc (relative to the number of photons immediately after transplantation = 100) was plotted against time, quantitative comparison of the extents of proliferation of the two cell lines with different metastasizing potentials was possible (Fig. 4C).

**Evaluation of the possibility of quantitative and objective screening of the effectiveness of anticancer agents.** In a previous study, tumor growth was found to be suppressed in animals given i.v. injections of CPT-11, resulting in a significant prolongation

**Figure 3.** Correlation between the photon counts and tumor volume. A, nude mice bearing 44As3Luc tumors in the s.c. were visualized in anesthetized animals after i.p. inoculation of luciferin. B, correlation plot; strong correlation ( $R = 0.97$ ) was observed between the conventional methods and our photon counting analysis method for monitoring the growth of a s.c. 44As3Luc tumor ( $n = 5$ ). The tumor mass was measured at predetermined time intervals in two dimensions with calipers, and the tumor volume was calculated according to the equation  $(l \times w^2) / 2$ , where  $l$  is the length and  $w$  is the width (16).





**Figure 4.** Quantitative photon counting analysis of progression process of peritoneal disseminated metastasis of the highly metastatic and the parent cell lines. *A*, detection of progression process of peritoneal disseminated metastasis. *B*, photon counting analysis of the peritoneal disseminations after orthotopic implantation (yellow arrow, site) of the cells. *C*, quantitative analysis of progression process of peritoneal disseminated metastasis of 44As3Luc (●) and HSC44Luc (▲) cell lines ( $n = 5$ ). This experiment was repeated thrice, and similar results were observed each time.

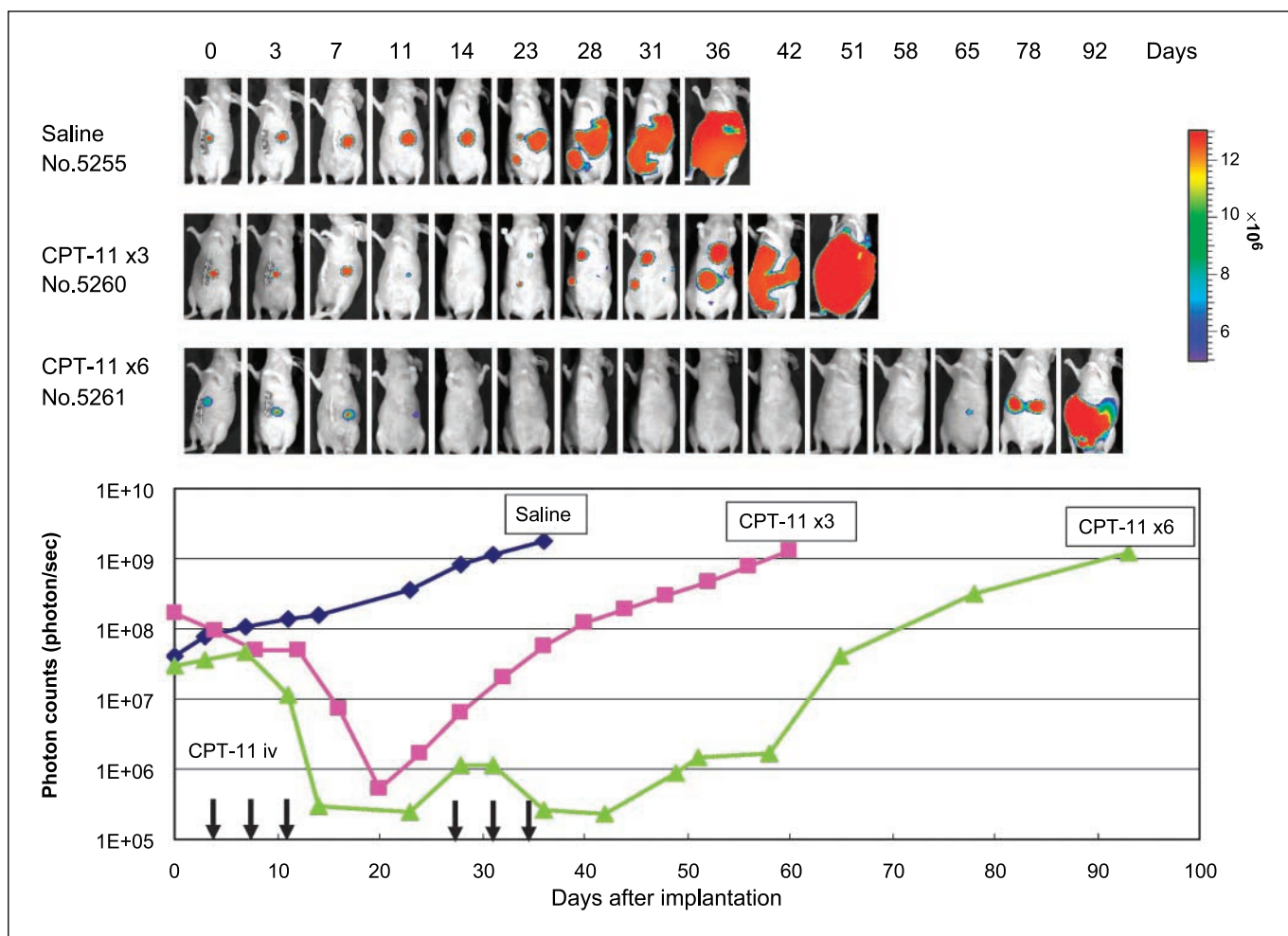
of the survival period (17). A similar evaluation was conducted in the present study using 44As3Luc cells. Figure 5 (*top*) shows a typical example of the photon counting analysis, whereas Figure 5 (*bottom*) shows the time course of the changes in the number of photons. Following three doses of CPT-11 (200 mg/kg/mouse), the tumor gradually decreased in size, reaching a level close to the limit of detection on the 20th day. During the 5th and 6th week, the tumor began to show slow growth in the stomach followed during the 8th/9th week by peritoneal invasion and the onset of cancerous peritonitis accompanied by ascites formation and death of the animals. The survival period was markedly longer in the drug-treated group compared with that in the saline-treated controls. Plotting of the number of photons measured (average of five animals) against time yielded a tumor growth curve, thus allowing quantitative evaluation of drug-induced suppression of the progression of peritoneal dissemination (Fig. 5, *bottom*).

As stated above, the 44As3Luc cells began to proliferate again during the 5th/6th week after implantation in the CPT-11 treatment group. We therefore gave three additional doses beginning on day 28 (after the onset of re-proliferation). Figure 5 (*top*) shows a typical example of the bioluminescence signal in

such a case. The additional doses of CPT-11 (400 mg/kg/mouse) markedly suppressed the proliferation of the 44As3Luc cells until around day 60; however, proliferation again began to be detected thereafter. By around day 80, the tumor started to grow more rapidly and spread, causing moribund animals to appear by around day 90. The survival period of the animals was markedly prolonged by the additional drug doses. Figure 5 (*bottom*) shows the time course of changes in the number of photons (average of five animals). Quantitative comparison of the proliferation and spread of the tumor cells was possible between the drug treatment group and the control group and between two drug treatment groups, thus allowing objective evaluation of the responses to treatment.

## Discussion

Before the present study, very little was known about how scirrhous gastric carcinoma cells invaded and proliferated within the primary lesion, how they exfoliated and thus became free, how they colonized and proliferated within the peritoneal cavity, or how they advanced to the stage of cancerous peritonitis. Herein, we investigated the course of proliferation and spread of gastric cancer



**Figure 5.** Quantitative photon counting analysis of the effect of CPT-11 on peritoneal disseminated metastasis 44As3Luc mouse model. Effects of CPT-11 in the peritoneal dissemination mouse model established using orthotopically implanted 44As3Luc cells. Mice receiving CPT-11 (arrow) or vehicle alone as control ( $n = 5$ ;  $P < 0.001$ ) were monitored twice weekly for the development of peritoneal dissemination. Similar results were obtained in a second experiment conducted independently.

cells by sacrificing the animals at different points of time after orthotopic implantation of the highly metastatic tumor cell line 44As3 (17) and conducted anatomic and histopathologic examinations in the sacrificed animals. In this experiment, the sequence of findings seems to endorse the previous contention that gastric cancer cells invade deeper layers of the gastric wall to reach the serosa and then exfoliate, thereby being released into the peritoneal cavity, resulting in peritoneal dissemination.

The growth of tumors in the gastric wall and the subsequent progression to cancerous peritonitis are difficult to monitor extracorporeally unlike s.c. tumors. For monitoring the progression of tumor dissemination, the only possible method was to implant the tumor cells into groups of mice and sacrifice the animals at different points of time for autopsy and observation; quantitative comparison was still not possible by this method (10–12, 18–25). All of these problems were resolved in the present study by introduction of the luciferase gene into tumor cells with a high metastasizing potential and subsequent *in vivo* photon counting analysis. In the first step, we confirmed that the results of the conventional method of evaluation in relation to proliferation of our gastric carcinoma cells were consistent with the results of our

photon counting analysis. We then conducted an experiment on a model of peritoneal dissemination. Using the *in vivo* photon counting technique, it was possible to observe the same animals successively, beginning from the growth of the tumor at the site of implantation to peritoneal dissemination and, finally, the formation of ascites. Furthermore, it was possible to observe the processes of dissemination progression on a real-time basis, allowing quantitative analysis and comparison of the course of proliferation and progression within the living body after implantation of a cell line with high metastasizing potential and its parent cell line based on changes in the photon number.

Needless to say, it is important to develop a screening model for exploring substances effective against tumors and ultimately developing clinically useful anticancer agents. We previously reported that an animal model of peritoneal dissemination established using the highly metastatic cell lines (44As3, 58As1, and 58As9) established by our group satisfied all of the requirements of a model for drug screening (17, 44). However, before this model can be applied as a universally valid drug evaluation system, the following problems must be resolved: (a) methods for appropriate observation and objective evaluation are urgently needed,

(b) excellent operative skill is indispensable for orthotopic implantation with high reproducibility, and (c) large numbers of animals are needed. With the establishment of this experimental system, the conventional problems associated with the evaluation of peritoneal dissemination have been overcome and highly reliable data are now obtainable. Therefore, a stage has been reached where this model of peritoneal dissemination can also be applied as a system for evaluation of the effects of drugs. Furthermore, because photon counting analysis allows noninvasive evaluation of the fate of cancer cells *in vivo* on a real-time basis, the pain experienced by experimental animals may be reduced, such that this technique would also be useful from the viewpoint of animal welfare (45).

We have used the bioluminescence signal from the luciferase reporter gene in our peritoneal metastasis model. Luciferase genes in our tumor cells can function stably over significant periods in tumors and in their metastases. To date, several other peritoneal metastasis models of human stomach cancer in animals have been reported (28, 31). For example, Hasegawa et al. (28) used green fluorescent protein (GFP) retroviral-infected human stomach cancer. In this nude mouse model, tumor cells were peritoneally injected and GFP transduction allowed visualization of the subsequent metastatic process. A major advantage of GFP labeling is that imaging requires no preparative procedures and hence allows for direct visualization in living tissue (26, 27, 29, 32, 34). In contrast, photon counting technique requires exogenous

injection of luciferin substrate, which can stress the animals, and in addition, the intensity of the luciferase signal may sometimes be variable and unstable (46). Furthermore, Ray et al. (32) reported that red fluorescent protein imaging is ~1,000 times stronger than that of luciferase *in vivo*. Therefore, for monitoring the tumor metastasis process at the single-cell level, fluorescence imaging may be the more practical method. In fact, fluorescence-based orthotopic metastatic models have been used to study mechanisms and for drug discovery (14, 30, 33, 35).

In conclusion, our photon counting analysis involving a highly metastatic cell line, 44As3Luc, seems to be a useful model for studies, such as those designed to clarify the mechanism of peritoneal dissemination progression in intractable scirrhous gastric carcinoma, and for the development of new agents effective against such tumors.

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