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Establishment of a novel method to evaluate peritoneal microdissemination and therapeutic effect using luciferase assay

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Key words

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Peritoneal dissemination is a major cause of recurrence in patients with malignant tumors in the peritoneal cavity. Effective anticancer agents and treatment protocols are necessary to improve outcomes in these patients. However, previous studies using mouse models of peritoneal dissemination have not detected any drug effect against peritoneal micrometastasis. Here we used the luciferase assay to evaluate peritoneal micrometastasis in living animals and established an accurate mouse model of early peritoneal microdissemination to evaluate tumorigenesis and drug efficacy. There was a positive correlation between luminescence intensity in *in vivo* luciferase assay and the extent of tumor dissemination evaluated by *ex vivo* luciferase assay and mesenteric weight. This model has advantages over previous models because optimal luciferin concentration without cell damage was validated and peritoneal microdissemination could be quantitatively evaluated. Therefore, it is a useful model to validate peritoneal micrometastasis formation and to evaluate drug efficacy without killing mice.

Peritoneal dissemination is a major cause of recurrence in patients with colorectal, gastric, pancreatic, and ovarian cancers and is associated with a poor prognosis.[1] To improve outcomes in patients with peritoneal dissemination, clinical studies are focusing on systemic i.v. chemotherapy and/or local i.p. chemotherapy.[2–10] However, ongoing investigation of novel drugs and treatment protocols is warranted.

A number of peritoneal dissemination models have been developed to evaluate drug efficacy and toxicity in living animals with peritoneal metastasis.[11–13] However, in these models, disseminated tumor cells cannot be observed macroscopically. Therefore, it is necessary to kill the model animals to evaluate tumor spread using mesenteric weight. To reduce the need of killing experimental animals, a novel model of peritoneal dissemination to evaluate tumor spread quickly and accurately in living animals is needed.

We used the luciferase assay to evaluate the peritoneal tumors in living animals. Luciferase-expressing cancer cells are luminous, and the luciferase assay is useful to detect living cancer cells in the peritoneal cavity with high sensitivity and reproducibility. Previous studies have reported the effectiveness of luciferase assay in macroscopically observable peritoneal tumors.[14–18] However, the assay has not been used to investigate undetectable peritoneal micrometastasis. We previously reported that the adhesion of cancer cells to the peritoneum occurs within 24 h[19,20] as the first stage of peritoneal dissemination. Therefore, if invisible peritoneal microdissemination at 24 h after tumor inoculation could be detected using luciferase assay, drug efficacy against peritoneal dissemination could be quickly and accurately assessed in *in vivo* mouse models.

In this study, we developed a novel method using luciferase assay to evaluate peritoneal microdissemination and drug efficacy in a mouse model. We validated the optimal luciferin concentration that did not cause cell damage and identified a positive correlation between luminescence in *in vivo* luciferase assay and the extent of tumor dissemination. With this method, it is possible to evaluate tumorigenesis quickly and accurately and at a low cost, with reduced need to kill experimental animals.

Materials and Methods

Cell line. Colon 26-luc cells, mouse rectal carcinoma cell lines transfected with pMSCV-luc, were kindly gifted by Dr. Murakami (Division of Bioimaging Sciences, Center for

Molecular Medicine, Jichi Medical University, Tochigi, Japan) in 2010. The cells were cultured in RPMI-1640 medium (Sigma-Aldrich, Tokyo, Japan) containing 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin (Pen strep; Gibco, Thermo Fisher Scientific, Tokyo, Japan), and 10 μg/mL puromycin (Sigma-Aldrich) at 37°C in a humid atmosphere with 5% CO_2.

Concentration of luciferin. *In vitro* and *in vivo* luciferase assays were carried out to determine the optimal administration concentration of luciferin (Ieda Trading, Tokyo, Japan). For the *in vitro* luciferase assay, 1.0×10^5 colon 26-luc cells in 50 μL medium were seeded into each well of a 96-well plate and incubated at 37°C for 24 h. Next, 50 μL/well luciferin was added at concentrations ranging from 0.008 mg/mL to 8.0 mg/mL, and images were captured after 10 min. For the *in vivo* luciferase assay, BALB/c mice were i.p. inoculated with 1.0×10^6 colon 26-luc cells in 0.5 mL PBS. On day 7, luciferin at 0.5, 1.0, or 1.5 mg/0.5 mL was injected and luminescence was observed.

Luciferin toxicity assay. To assess the toxicity of luciferin by WST-8 assay, 1.0×10^4 colon 26-luc cells in 100 μL medium were seeded into each well of a 96-well plate and incubated at 37°C for 24 h. Next, 50 μL/well luciferin at 0.25, 0.5, 1.0, 2.0, or 4.0 mg/mL was added. Sixty minutes later, luciferin was removed and culture medium (RPMI-1640) was added, and the plate was incubated at 37°C for 24 h. Cell viability was determined using a cell counting kit-8 (Dojindo, Kumamoto, Japan). Cell viability was determined by measuring the absorbance of the cells at 450 nm with the reference wavelength at 650 nm, using a Multiskan FC microplate reader (Thermo Fisher Scientific, Tokyo, Japan).

Animals. Inbred female BALB/cCrSlc mice (Japan SLC, Shizuoka, Japan) were obtained at 5 weeks of age and maintained under specific pathogen-free conditions. They were used for experiments at 6–7 weeks of age. All experiments and procedures for care and treatment of animals in this study were carried out in accordance with the requirements of the Gunma University Animal Care and Experimentation Committee (Experimental Protocol No.14-026) (Gunma University, Maebashi, Japan).

Intraperitoneal microdissemination model. As described in our previous studies, peritoneal dissemination was investigated in mice models.[11,19] Twenty BALB/c mice (nos. 1–20) were i.p. inoculated with 1.0×10^6 colon 26-luc cells in 0.5 mL PBS (day 0). On tumor implantation, cells were injected in the left lower abdomen of mice, and needles were replaced in each mouse. Twenty-four hours after injection (day 1), microdissemination was observed by *in vivo* luciferase assay. Mice with luminescence observed through the intact abdominal wall, as described below, were randomly divided into three groups: no treatment group, cisplatin (CDDP, 10 mg/kg; Sigma-Aldrich) treated group, and gemcitabine (240 mg/kg; Eli Lilly, Hyogo, Japan) treated group. Both CDDP and gemcitabine in PBS were i.p. injected into the mice on day 1. Body weights were measured on days 1, 3, 7, and 10.

***In vivo* luciferase assay and setting.** *In vivo* peritoneal microdissemination was examined using the OptimaShot CL-420α chemiluminescence imaging system (Wako, Osaka, Japan). On day 1, model mice were anesthetized with 2–5% isoflurane (Abbott Japan, Tokyo, Japan) in a custom-made box (Alfabio, Gunma, Japan; Fig. S1), and 0.5 mg luciferin (0.5 mg/mL) was injected i.p. Up to 12 mice could be placed in the box at the same time but the mice were separated by partitions to prevent interference from luminescence of neighboring mice. Luciferase assay was started within 5 min of injection of luciferin and luminescence from microdissemination was captured for 20 min. The assay was repeated on day 10, and luminescence was captured for 10 min. Luminescence intensity was calculated using Image J software (Rasband, W.S., U. S. National Institutes of Health, Bethesda, MD, USA).

***Ex vivo* luciferase assay and setting.** On day 10, *ex vivo* luciferase assay and mesenteric weight measurement were carried out to evaluate the accuracy of the *in vivo* luciferase assay. Following the *in vivo* luciferase assay, mice were killed by injuring the abdominal aorta under sufficient anesthesia. Upper, lower, and left side abdominal walls were dissected and the gastrointestinal tract from the esophagus to the rectum, and liver were resected. *Ex vivo* luciferase assay was immediately started following dissection, and luminescence was observed in four areas, the gastrointestinal tract and mesenterium, abdominal wall, retroperitoneum, and liver (Fig. S2). Luciferin 1.0 mg (1.0 mg/mL) was equally applied to the four areas and then luminescence was captured for 10 min. The number of luminescent spots was counted. Finally, the mesenteric weights were measured.

Statistical analysis. When the results of ANOVA were significant, Dunnett's multiple comparison tests were used to assess differences in luminescence intensity, numbers of luminescent spots, and body weight among the three groups. The statistical correlation between luminescence intensity, numbers of luminescent spots, and mesenteric weight was tested using Spearman's correlation coefficient. All differences were considered statistically significant if $P < 0.05$. Statistical analyses were carried out using the JMP 5 for Windows software package (SAS Institute, Tokyo, Japan).

Results

Optimal luciferin concentration was determined by *in vitro* and *in vivo* luciferase assay. *In vitro* luciferase assay showed that optimal luminescence was observed at 0.5 mg/mL (Fig. 1a). In *in vivo* luciferase assay, luminescence intensity was similar at each injection dose of luciferin (0.5, 1.0, and 1.5 mg; Fig. 1b). Cell viability was inhibited at luciferin concentrations ≥1.0 mg/mL (Fig. 1c).

Evaluation of tumor transplantation by *in vivo* luciferase assay. In *in vivo* luciferase assay, on day 1, luminescence was observed through the intact abdominal wall in 13/20 mice (65%; Fig. 2). Mice with luminescence were randomly divided into three groups. The no treatment group included mouse no. 7, 9, 10, and 17; the CDDP (10 mg/kg) group included mouse no. 4, 6, 16, and 18; and the gemcitabine (240 mg/kg) group included mouse no. 3, 8, 12, 13, and 14. On day 10, all mice with luminescence on day 1 had peritoneal dissemination validated by pathological findings, *in vivo* luciferase assay, and *ex vivo* luciferase assay. Mouse no. 10 mouse died of peritoneal dissemination on day 9.

On day 10, seven mice with no luminescence on day 1 were reexamined. Luminescence was observed in 5/7 (71.4%) of those mice (no. 1, 5, 11, 19, and 20; Fig. S3). Mouse no. 5 died on day 10; no. 11 died on day 21; and no.1, 19, and 20 died on day 24. Mouse no. 2 and 15 did not have luminescence on day 10, but mouse no. 15 died on day 28. A tumor nodule was observed in her left lower abdominal wall. On day 30, *in vivo* luciferase assay was repeated in mouse no. 2. No luminescence was observed, and no tumor nodules were observed on dissection. Therefore, the final tumor implantation rate of colon 26-luc cells was 95% (19/20).

Evaluation of drug efficacy by *in vivo* luciferase assay. In *in vivo* luciferase assay, on day 10, luminescence was observed in all three of the surviving mice in the no treatment group, in

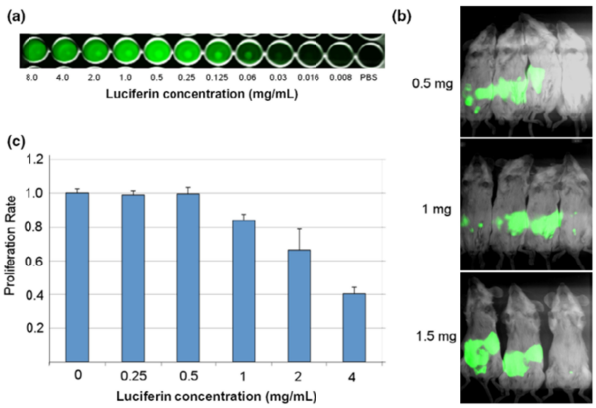

Fig. 1. Determination of the optimal concentration of luciferin for luciferase assay. (a) *In vitro* luciferase assay with concentrations of luciferin ranging from 0.008 to 8.0 mg/mL. Optimal luminescence was observed at 0.5 mg/mL. (b) *In vivo* luciferase assay in peritoneal dissemination model mice. Luminescence intensity was similar following i.p. injection of 0.5, 1.0, and 1.5 mg luciferin. (c) WST-8 assay showed that cell proliferation was inhibited at luciferin concentrations ≥1.0 mg/mL.

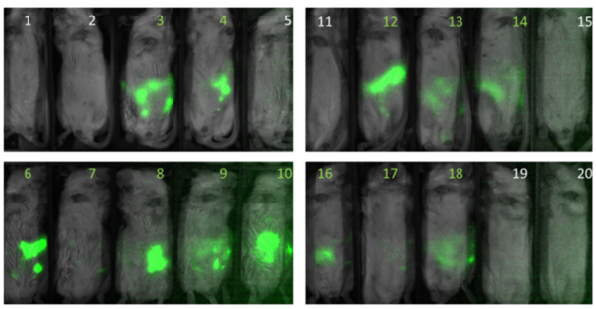

Fig. 2. *In vivo* luciferase assay to evaluate peritoneal dissemination on day 1. Mice were anesthetized with 2–5% isoflurane, and 0.5 mg luciferin was injected i.p. Luciferase assay was carried out within 5 min of injection, and images were captured after 20 min. Luminescence was observed in mouse no. 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, and 18.

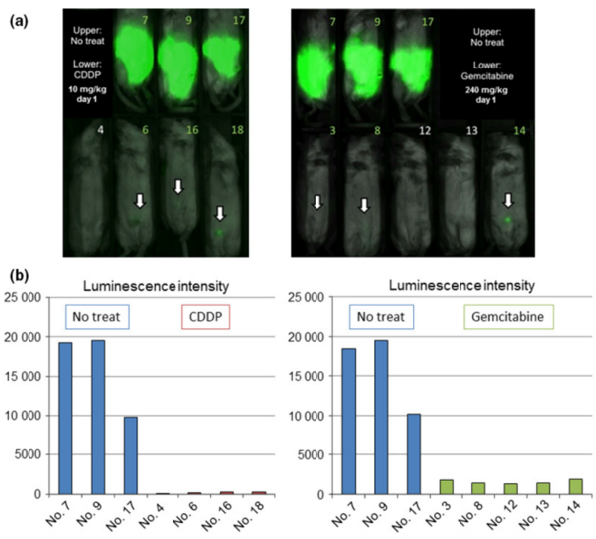

Fig. 3. *In vivo* luciferase assay to evaluate drug efficacy on day 10. (a) Intense luminescence was observed in the no treatment (no treat) group. Mouse no. 10 in this group died on day 9. White arrows show areas of luminescence. In the cisplatin (CDDP) group, luminescence was observed in mouse no. 6, 16, and 18 but not in mouse no. 4. In the gemcitabine group, luminescence was observed in mouse no. 3, 8, and 14. (b) Luminescence intensity was calculated by Image J software. Luminescence intensity was significantly stronger in the no treatment group than that in the CDDP and gemcitabine groups.

three of the four mice in the CDDP group (Fig. 3a), and in three of the five mice in the gemcitabine group. Luminescence intensity in the no treatment group was significantly stronger than that in the CDDP and gemcitabine groups (Fig. 3b).

Evaluation of drug efficacy by *ex vivo* luciferase assay. *Ex vivo* luciferase assay was carried out on day 10 to evaluate the efficacy of i.p. injected anticancer agents. Luminescent spots were observed in all four areas in all three groups (Fig. 4a, Table 1). However, the total number of spots was significantly higher in the no treatment group than that in both the CDDP and gemcitabine groups. On evaluation by area, there were significantly more luminescent spots in the no treatment group

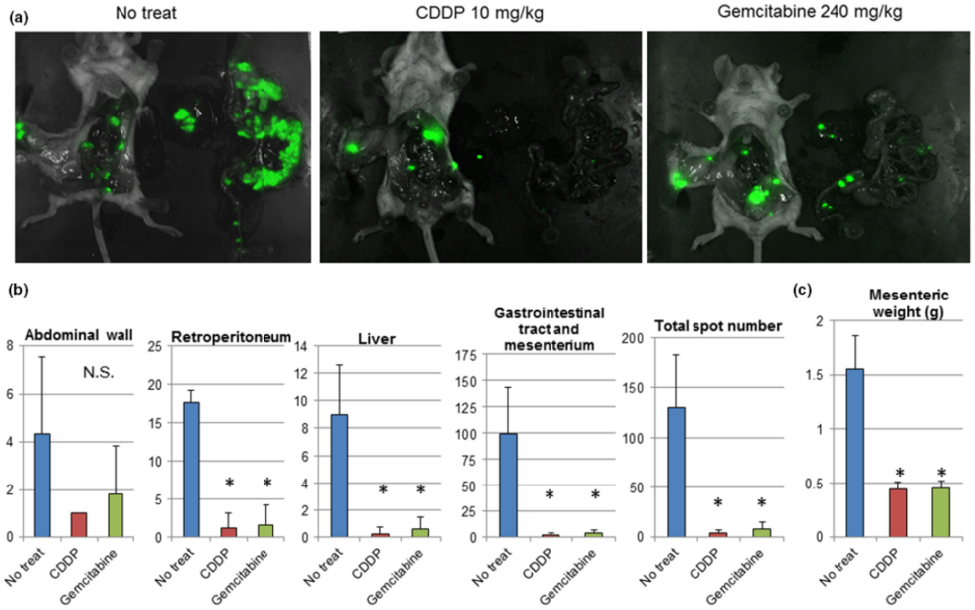

Fig. 4. *Ex vivo* luciferase assay on day 10. (a) Mice were killed under sufficient anesthesia. The abdomen was opened and divided into four areas: the abdominal wall, retroperitoneum, liver, and gastrointestinal tract and mesenterium. Luciferin (1.0 mg) was applied evenly over the four areas. Luminescence was captured for 10 min. Luminescent spots were observed in all areas in all groups. Compared with the no treatment (no treat) group, luminescent spots were reduced in groups treated with cisplatin (CDDP) or gemcitabine. (b) The number of luminescent spots was counted in each area in each group. In groups treated with CDDP or gemcitabine, the total number and the number of luminescent spots in all areas except the abdominal wall area were significantly reduced compared with the no treatment group. (c) Mesenteric weight was significantly heavier in the no treatment group than that in the CDDP and gemcitabine groups. *$P < 0.05$. N.S., not significant.

Table 1. Number of luminescent spots in each treatment group of mice, by area and in total in *ex vivo* luciferase assay and mesenteric weights, to evaluate peritoneal microdissemination and therapeutic effect

No.	Treatment	Abdominal wall	Retroperitoneum	Liver	Gastrointestinal tract and mesenterium	Total	Mesenteric weight, g
7	No treat	3	18	8	90	119	1.9
9	No treat	8	19	13	147	187	1.44
17	No treat	2	16	6	60	84	1.32
4	CDDP	1	0	0	0	1	0.38
6	CDDP	1	4	0	1	6	0.53
16	CDDP	1	1	1	4	7	0.4
18	CDDP	1	0	0	0	1	0.46
3	Gemcitabine	4	6	2	7	19	0.35
8	Gemcitabine	1	2	1	4	8	0.47
12	Gemcitabine	0	0	0	1	1	0.42
13	Gemcitabine	4	0	0	6	10	0.54
14	Gemcitabine	0	0	0	1	1	0.47

Mice were grouped according to treatment: no treatment (no treat); 10 mg/kg cisplatin (CDDP); and gemcitabine 240 mg/kg.

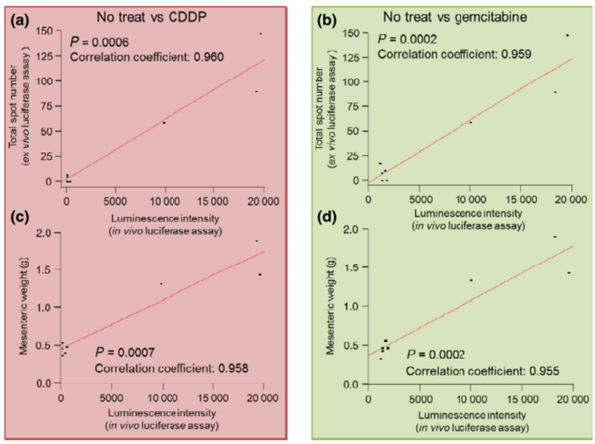

Fig. 5. Positive correlation of luminescence intensity with total luminescent spot number and mesenteric weight. (a, B) In each group, luminescence intensity in *in vivo* luciferase assay was positively correlated with the total number of luminescent spots in *ex vivo* luciferase assay. (c, d) In each group, luminescence intensity was positively correlated with mesenteric weight. CDDP, cisplatin; No treat, no treatment.

compared with those in the treatment groups for all areas except the abdominal wall (Fig. 4b). Mesenteric weight was significantly heavier in the no treatment group than that in the treatment groups (Fig. 4c). There was no significant difference in body weight among the three groups at any time point (Fig. S4).

Luminescence intensity positively correlated with total number of luminescent spots and mesenteric weight. In each group, there was a positive correlation between luminescence intensity in *in vivo* luciferase assay and the total number of luminescent spots in *ex vivo* luciferase assay (Fig. 5a,b). Furthermore, there was a positive correlation between luminescence intensity in *in vivo* luciferase assay and mesenteric weight (Fig. 5c,d) and between the total number of luminescent spots in *ex vivo* luciferase assay and mesenteric weight.

Discussion

In this study, we developed a novel peritoneal microdissemination mouse model using luciferase assay. This model has a number of advantages over previously reported mouse models of peritoneal dissemination. First, the optimal luciferin concentration that reduced cell viability was validated for *in vivo* luciferase assay. Second, peritoneal microdissemination could be quantitatively evaluated using *in vivo* luciferase assay. Finally, the model showed a positive correlation between luminescence in *in vivo* luciferase assay and the extent of tumor spread evaluated by *ex vivo* luciferase assay and mesenteric weight.

Previous studies have used 2.0–5.0 mg luciferin i.p. injected for *in vivo* luciferase assay to evaluate tumor extent in the peritoneal cavity.[14–18] Luciferin is thought to have low cytotoxicity for peritoneal tumor clusters analyzed by luciferase assay.[21] However, it remained unclear whether luciferin had a cytotoxic effect on colon 26-luc cells. Therefore, we investigated the optimal concentration and found that the dose of luciferin used in previous studies reduced cell viability in colon 26-luc cells. An i.p. injection of 0.5 mg luciferin was sufficient to produce optimal luminescent intensity.

We focused on the detection of macroscopically invisible peritoneal microdissemination using luciferase assay. The diameter of peritoneally disseminated tumors is usually heterogeneous. Therefore, small clusters of cancer cells might be affected by the cytotoxic effect of the high concentrations of luciferin used in previous models. We believe that the model developed in the present study can more accurately evaluate the efficacy of therapeutic drugs, particularly for peritoneal micrometastasis.

The transplantation rate of colon 26-luc cells by i.p. inoculation was 95% in this study. Although this was sufficient, a rate of 100% is ideal. Experimental bias relating to tumor implantation is one reason why large numbers of experimental animals are needed for *in vivo* studies of anticancer drug efficacy. To address this problem, it is important to identify which experimental animals have successfully been implanted with peritoneal tumors. Therefore, we used the *in vivo* luciferase assay not only to evaluate anticancer drug efficacy but also to identify successfully tumor-implanted mice for the study. *In vivo* luciferase assay before treatment was useful to confirm the engraftment of invisible micrometastasis in a setting where tumor clusters could not be detected by conventional methods, such as macroscopic observation. This strategy resulted in a transplantation rate of 100% in mice that were included in the experiment and provides an effective approach to reduce the number of laboratory animals for drug efficacy assays.

In previous studies, tests of drug efficacy were carried out after day 14.[11,19] We i.p. injected anticancer agents on day 1, and evaluated drug efficacy on day 10 using luciferase assay and mesenteric weight. Therefore, our mouse model reduced the required experimental period with confirmation of tumor engraftment on day 1 and assessment of microdissemination on day 10.

First, we validated the correlation between *in vivo* luciferase activity and peritoneal tumor extent as determined by mesenteric weight and the number of luminescent spots in *ex vivo* luciferase assay. This is very important data to confirm the accuracy and usefulness of experimental mouse models of peritoneal dissemination. However, few previous studies have investigated whether luminescent intensity viewed through the intact abdominal wall could be used to evaluate the extent of peritoneal dissemination. Therefore, our

method to quantitatively evaluate peritoneal dissemination *in vivo* provides an important advance over previous methods using luciferase assay. Incidentally, in *ex vivo* luciferase assay, the number of luminescent spots in the abdominal wall area did not significantly reduce in the treatment group. Based on the luminescence in this area, abdominal wall metastasis may be mixed as well as peritoneal dissemination. Therefore, it was suggested that enough efficacy against abdominal wall metastasis was not provided by i.p. administration of drugs.

Most previous studies have used the *in vivo* imaging system (IVIS) to evaluate luminescence.[14–17] The IVIS system is widely used for *in vivo* imaging by luciferase assay because of its high sensitivity, high throughput, and high resolution. However, this system is expensive for individual laboratories. In this study, we used the lower-priced OptimaShot CL-420α system and a custom-made box for 12 mice. This makes the model applicable to a wide range of experimental settings.

In summary, we established a novel method to evaluate tumorigenesis and drug efficacy using a mouse model of peritoneal microdissemination and luciferase assay. New drugs and treatment protocols to treat local dissemination will improve outcomes in patients with tumors in the peritoneal cavity. This model is useful to validate peritoneal micrometastasis formation and to evaluate drug efficacy with high accuracy, reduced experimental costs, and reduced the need to kill experimental animals.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Custom-made box used for luciferase assay.

Fig. S2. Assessment of *ex vivo* luciferase assay in each organ area: the abdominal wall, retroperitoneum, liver, and gastrointestinal tract and mesenterium.

Fig. S3. *In vivo* luciferase assay on day 10 in mice with no luminescence on day 1.

Fig. S4. Body weight change among the three groups.