A Mouse Model of Fluorescent Protein-expressing Disseminated Peritoneal Lymphoma for Fluorescence-guided Surgery

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Abstract. Background: Fluorescence-guided surgery (FGS) of tumors is an area of intense development. Peritoneally-disseminated cancer, however, represents a difficult surgical challenge. Materials and Methods: To help meet this challenge, EL4 mouse T-cell lymphoma cells expressing red fluorescent protein (RFP) were injected intraperitoneally in nude mice. Results: Two weeks after injection of EL4-RFP cells, established peritoneally-disseminated tumors were observed. FGS was performed using a hand-held portable fluorescence imaging system (Dino-Lite). FGS enabled detection of very small peritoneal disseminated tumors and completely resected them in contrast to bright-light which only partially detected the tumors. Conclusion: The present report indicates the feasibility of FGS of peritoneally-disseminated cancer.

Our laboratory has developed fluorescence-guided surgery (FGS) in mouse models of cancer (1). FGS on orthotopic nude-mouse models of colon cancer (2-6), pancreatic cancer (7-19), lung cancer (20), sarcoma (21), glioma (22) and liver metastasis (5, 6, 23) has been shown to have significant improvement over bright-light surgery (BLS). A small hand-held fluorescence imaging device has recently been used for FGS in orthotopic mouse models (4, 13) that can be used in the clinic. The mouse models used thus far for FGS have also included patient-derived orthotopic xenograft (PDX) models (3, 4, 13, 15, 16, 18, 19) in nude mice.

Recently, we have developed a model of the EL-4 mouse lymphoma expressing red fluorescent protein (EL4-RFP). A syngeneic color-coded imageable lymphoma model was previously established to visualize recruitment of host stromal cells by malignant lymphoma during metastasis. EL4-RFP cells were injected into the tail vein of C57/BL6-GFP transgenic mice. EL4-RFP metastasis was observed in multiple sites, which were also enriched in host GFP-expressing cells. Furthermore, EL4-RFP lymphoma cells were also observed along with host GFP stromal cells in the peripheral blood and bone marrow, as well as the liver (24).

RFP-expressing EL4 lymphoma cells were also previously injected subcutaneously in C57/BL6 GFP transgenic mice and formed tumors by 35 days after cell transplantation. Using the Dino-Lite hand-held, portable fluorescence imaging system, subcutaneous tumors including the tumor microenvironment (TME) were clearly visualized and resected. In the resected tumor, GFP-expressing host stromal cells, including adipocyte-like cells, and blood vessels containing lymphocytes were observed by color-coded confocal microscopy to be closely associated with the lymphoma cells (25).

The present report describes successful FGS of peritoneally-disseminated EL-4-RFP, a model of highly-challenging cancer surgery.

Materials and Methods

Mouse experiments. All experiments were conducted in accordance with the institutional guidelines of Gifu University, Gifu, Japan, and approved by the animal research committee and the committee on living modified organisms of Gifu University. In order to minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments. Animals were anesthetized by intramuscular injection of a 0.02 ml solution of 100 mg/kg ketamine. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The use of
Figure 1. Time course of fluorescence-guided surgery (FGS) of peritoneal-disseminated lymphoma. Pre-operative and time-course intra-operative images are shown. Left panels show bright-field images and right panels are fluorescence images of peritoneally-disseminated tumors obtained with the Dino-Lite hand-held fluorescence scope (bar=5 mm). Under bright light, tumors were very difficult to distinguish. In contrast, under fluorescence, tumors were clearly visualized as indicated (arrows). As FGS proceeded, tumors were removed according to their number from 1 to 5.
animals was necessary to develop FGS of peritoneal metastasis. Animals were housed with no more than 5 per cage, in a barrier facility on a high efficiency particulate arrestance (HEPA)-filtered rack under standard conditions of 12-hour light/dark cycles. Seven-week-old BALB/c-nu/nu nude mice (Charles River Laboratories, Inc., Yokohama, Japan) were used in this study. Mice were kept in a barrier facility under HEPA filtration (as noted above). Mice were fed with an autoclaved laboratory rodent diet. All mouse surgical procedures and imaging were performed with the animals anesthetized by subcutaneous injection of the ketamine mixture described above.

Cell line and culture condition. EL-4, a mouse lymphoma cell line, was established from a lymphoma induced in a C57BL mouse by 9,10-dimethyl-1,2-benzanthracene (DMBA). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin (Gibco-BRL, Grand Island, NY, USA). The cells were cultured at 37°C in a 5% CO₂ incubator (25).

Red fluorescent protein (RFP) transduction of lymphoma cells. EL4 lymphoma cells were labeled with RFP as previously reported (24) using retroviruses expressing RFP. EL-4 cells were transfected with retroviruses.

EL4-RFP peritoneally-disseminated lymphoma model. Seven-week-old BALB/c-nu/nu nude mice were used as the host for EL4-RFP lymphoma cells. EL4-RFP lymphoma cells were first harvested and washed three times with cold serum-free medium and then re-suspended with serum-free RPMI 1640 medium. RFP EL4
lymphoma cells (1×10⁶ in 100 μl serum-free medium) were injected intraperitoneally in nu/nu (nude) mice. Two weeks after injection, peritoneal metastasis was confirmed by laparotomy.

**FGS of peritoneally-disseminated lymphoma.** Two weeks after intraperitoneal administration of EL4-RFP, peritoneally-disseminated lymphoma was observed. FGS was performed using the Dino-Lite imaging system (AM4113TYFGW Dino-Lite Premier; AnMo Electronics Corporation, Hsinchu, Taiwan) (4, 13).

**Results and Discussion**

**FGS resection of peritoneally-disseminated lymphoma.** We performed FGS of peritoneally-disseminated lymphoma demonstrated by pre-operative and time-course intra-operative imaging (Figure 1). The resection of each peritoneally-disseminated tumor was recorded in real time with the Dino-Lite (Figure 1). The tumors were removed with minimal organ injury and with maximal resection margins.

Resected tumors are shown in bright-field and under fluorescence (Figure 2). Tumor margins are sharply visualized with the Dino-Lite with very little autofluorescence signal. Under bright light, tumor margins were unclear (Figures 1, 2).

Histopathological evaluation of margins of the resected tumors are shown in Figure 3. Tumors removed by FGS contained abundant cancer-associated stromal cells, surrounded by normal tissues. Viable cancer cells were not detected in the resected margins. This result shows the precision of FGS with the portable hand-held Dino-Lite on even very small tumor nodules containing abundant host stromal cells. This technique allowed maximal tumor resection with minimal or no residual lymphoma.

Tumors are often obscure in the normal background, difficult to distinguish and overlooked during surgery. Cancer cells labeled with fluorescence can be accurately detected and entirely removed during surgery. Even peritoneally-disseminated cancer, a particularly difficult surgical challenge, can be successfully resected by FGS, as demonstrated in the present report.

**Conclusion**

FGS has been shown to detect tiny peritoneally-disseminated lymphoma and completely resect them. The results of the present study suggest that FGS has clinical potential for peritoneally-disseminated cancer. The pioneering studies of our laboratories have demonstrated two strategies for labeling tumors for FGS: a telomerase-dependent adenovirus containing GFP (26) and a fluorescent conjugated tumor-specific antibody (27). FGS in now emerging in the clinic (28-30) and should become the standard paradigm in the near future.

**Conflicts of Interest**

None of the Authors have any conflict of interest with regard to this study.

**Dedication**

This paper is dedicated to the memory of A.R. Moossa, MD and Sun Lee, MD.

**References**


4486


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