

Imageable Fluorescent Metastasis Resulting in Transgenic GFP Mice Orthotopically Implanted with Human-patient Primary Pancreatic Cancer Specimens

ATSUSHI SUETSUGU^{1,2,4}, MATTHEW KATZ³, JASON FLEMING³, MARK TRUTY³, RYAN THOMAS³, SHIGETOYO SAJI⁴, HISATAKA MORIWAKI⁴, MICHAEL BOUVET² and ROBERT M. HOFFMAN^{1,2}

¹AntiCancer, Inc. San Diego, CA, U.S.A.;

²Department of Surgery, University of California, San Diego, CA, U.S.A.;

³Department of Surgical Oncology, MD Anderson Cancer Center, Houston, TX, U.S.A.;

⁴Gifu University Graduate School of Medicine, Gifu, Japan

Abstract. Tumors from pancreatic cancer patients were established in NOD/SCID mice immediately after surgery and subsequently passaged orthotopically in transgenic nude mice ubiquitously expressing green fluorescent protein (GFP). The primary patient tumors acquired GFP-expressing stroma. Subsequent liver metastases, and disseminated peritoneal metastases maintained the stroma from the primary tumor, and possibly recruited additional GFP-expressing stroma, resulting in their very bright fluorescence. The GFP-expressing stroma included cancer-associated fibroblasts and tumor-associated macrophages in both the primary and metastatic tumors. This imageable model of metastasis from a patient-tumor is an important advance over patient "tumorgraft" models currently in use, which are implanted subcutaneously, do not metastasize and are not imageable. The new imageable model of patient pancreatic cancer metastasis provides unique opportunities to identify current and novel antimetastatic therapeutics for individual patients.

Our laboratory pioneered surgical orthotopic implantation (SOI) metastatic nude-mouse models using patient tumor specimens in the early 1990s (1, 2). These orthotopic models of patient tumors are more patient-like than ectopic subcutaneous models of patient tumors. Human tumor specimens have been grown *in vivo* since 1969 with the introduction of the T-cell-deficient nude mouse (3). In the 1970s and 1980s, many laboratories worldwide successfully

grew human tumors in nude mice (4-12). Recently, a number of laboratories have reported on human patient tumors implanted in immunodeficient mice (13-19). These models have now been given names such as "tumorgraft" (20) and "xenopatients" (21); however, they remain ectopic subcutaneous models, as were the models of the 1970s and 1980s described above, and, therefore, not patient like.

We established the principle of making patient tumors imageable by implanting them in transgenic nude mice expressing fluorescent proteins (22). The primary tumors acquired fluorescent stroma expressing a fluorescent protein derived from the transgenic host. Fluorescent stroma persisted in the primary tumor for at least two passages. These results predicted that metastases resulting from the fluorescent primary tumors would retain their stroma and also become fluorescent and imageable, which is the topic of the present report.

Materials and Methods

Mice. Transgenic C57/B6-GFP mice (23) were obtained from AntiCancer, Inc. (San Diego, CA). The C57/B6-GFP mice expressed the *Aequorea victoria* (GFP) under the control of the chicken β -actin promoter and cytomegalovirus enhancer. All of the tissues from this transgenic line, with the exception of erythrocytes and hair, fluoresced green under excitation light. The GFP gene, regulated as described above, was crossed into nude mice on the C57/B6 background (24). NOD/SCID mice were purchased from Charles River (Wilmington, MA, USA).

Animal care. Transgenic GFP nude mice (25) were bred and maintained in a HEPA-filtered environment at AntiCancer Inc. with cages, food and bedding sterilized by autoclaving. The animal diets were obtained from Harlan Teklad (Madison, WI, USA). Ampicillin (5.0%, W/V; Sigma, St. Louis, MO, USA) was added to the autoclaved drinking water.

All surgical procedures and imaging were performed with the animal anesthetized by intramuscular injection of 0.02 ml of a solution of 50% ketamine, 38% xylazine and 12% acepromazine

Correspondence to: Robert M. Hoffman, Ph.D., Anti Cancer, Inc., 7917 Ostrow Street, San Diego, CA 92111, U.S.A. Tel: +1 8586542555, Fax: +1 8582684175, email: all@anticancer.com

Key Words: Human-patient pancreatic cancer, nude mice, metastasis, GFP, stroma, tumor, microenvironment, imaging.

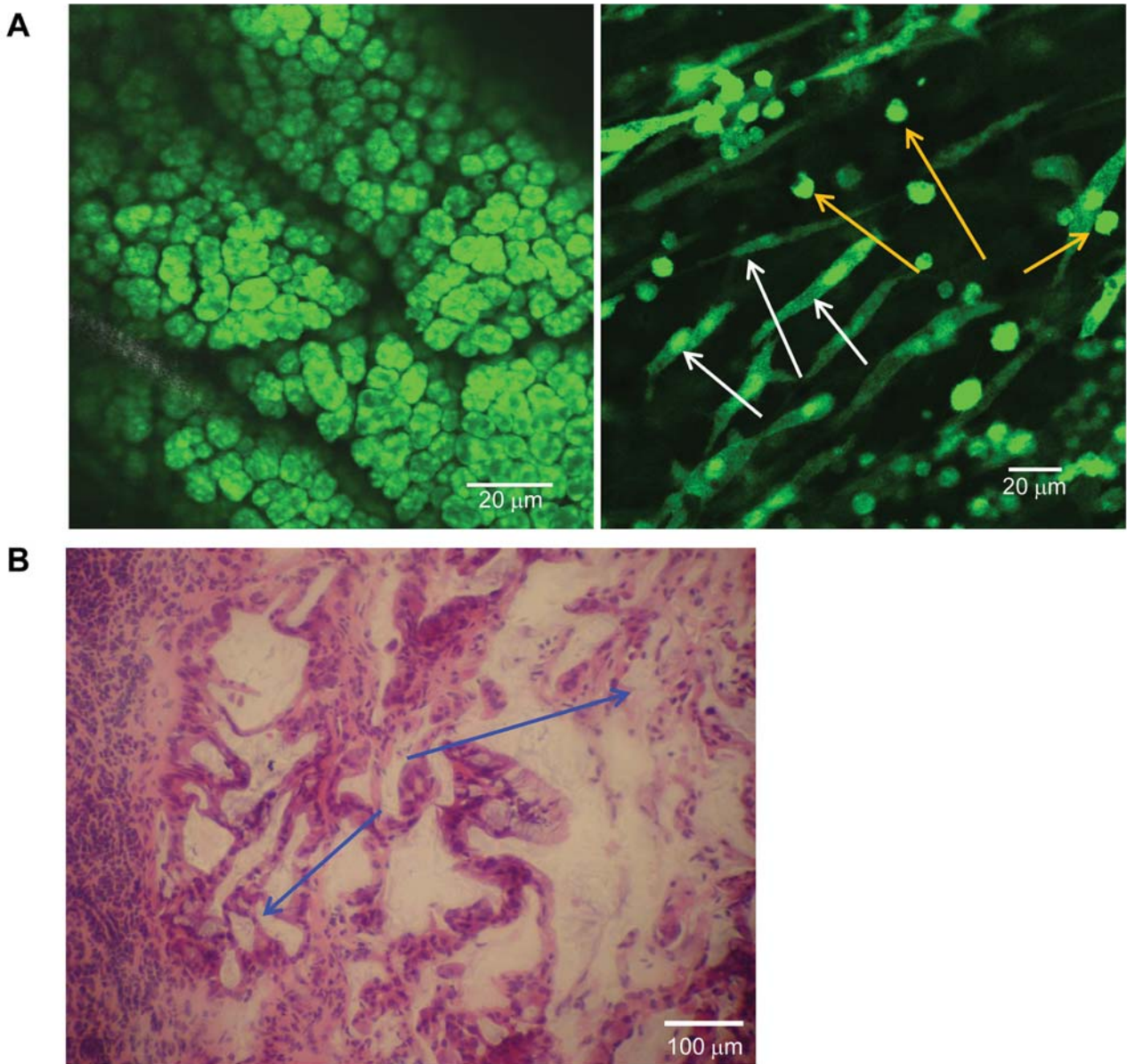


Figure 1. A: Left panel shows host green fluorescent protein (GFP)-expressing mouse pancreas. Right panel shows primary tumor image. Yellow arrows indicate tumor-associated macrophages (TAMs). White arrows indicate cancer-associated fibroblasts (CAFs). Images were taken with an Olympus FV1000 scanning laser microscope. Bar=20 μm. B: Excised primary tumor was stained with H&E. Blue arrows indicate pancreatic tubular adenocarcinoma. Bar=100 μm.

maleate. All animal studies were conducted in accordance with the principles of and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance number A3873-1.

Establishment of tumorgraft model (F1) of pancreatic-cancer patient tumors. Pancreatic-cancer tumor tissue from patients was obtained at surgery with informed consent and cut into 3-mm³ fragments and transplanted subcutaneously into NOD/SCID mice (18, 19).

Orthotopic tumorgraft (F2) of pancreatic-cancer patient tumor in transgenic GFP nude mice. The F1 tumors from NOD/SCID mice were harvested and cut into 3 mm³ fragments and transplanted orthotopically into six-week-old transgenic nude GFP mice (F2 model).

Fluorescence imaging. After 110 days, tumors were initially imaged with an OV100 Small Animal Imaging System (Olympus, Tokyo, Japan). The resected primary tumor, liver metastases and disseminated

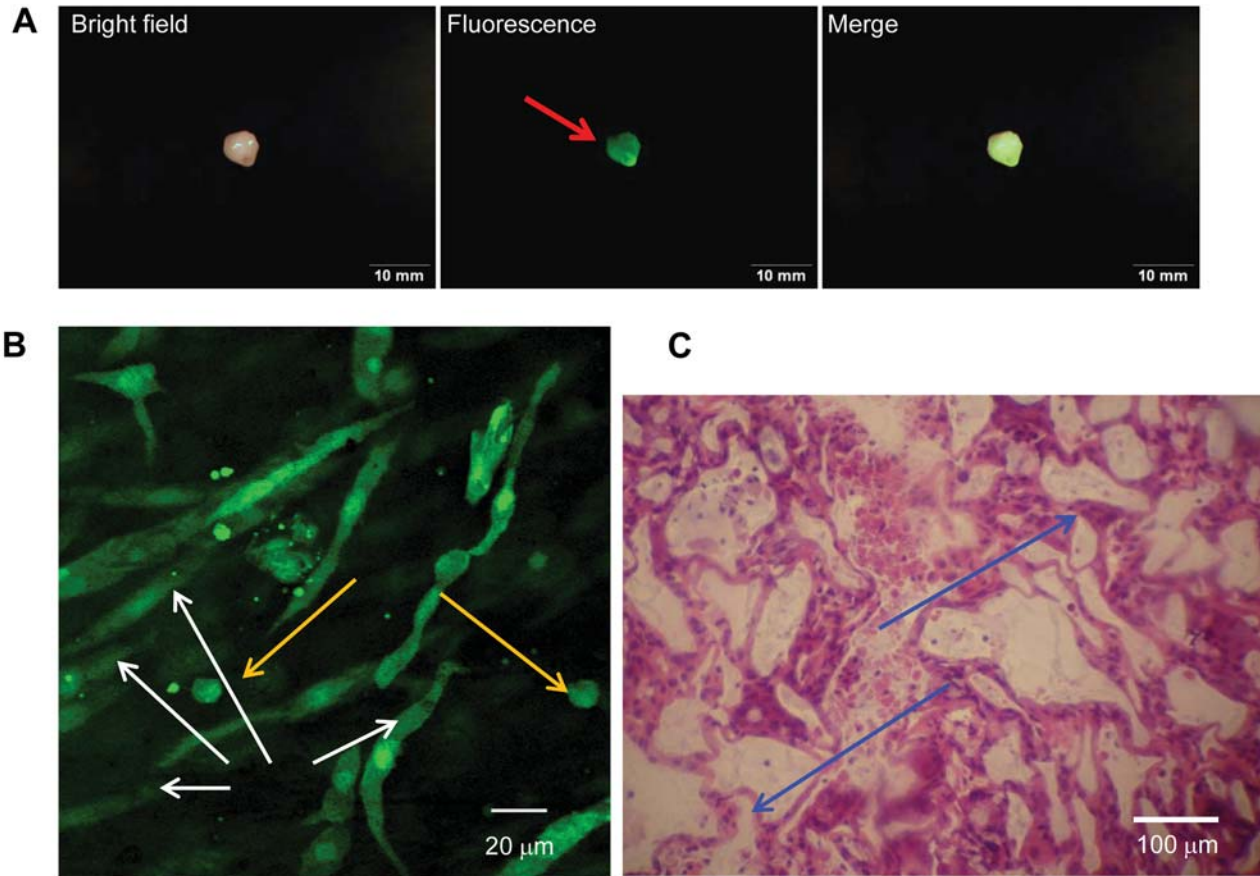


Figure 2. A: Image of disseminated peritoneal metastasis. Red arrow indicates disseminated peritoneal metastasis with green fluorescent protein (GFP) stroma. Bar=10 mm. B: Image of disseminated peritoneal metastasis. Yellow arrows indicate tumor-associated macrophages (TAMs). White arrows indicate cancer-associated fibroblasts (CAFs). Image was taken with an Olympus FV1000 confocal laser microscope. Bar=20 μ m. C: Disseminated peritoneal metastasis stained with H&E. Blue arrows indicate pancreatic tubular adenocarcinoma. Bar=100 μ m.

peritoneal metastases were observed with an FV1000 confocal microscope (Olympus).

Histological analysis. The primary tumor, liver metastases and disseminated peritoneal metastases were sectioned at a thickness of 8 μ m, and stained with hematoxylin and eosin for microscopic analysis.

Results

Engraftment of tumors from pancreatic cancer patients (F1) in NOD/SCID mouse. Tumors from patients with pancreatic cancer were initially transplanted subcutaneously into NOD/SCID mice within two hours of surgery. Tumors were detected by day 30 (18, 19). Tumors were then harvested from the NOD/SCID mice and cut into 3-mm³ fragments.

GFP host stromal cells infiltrate orthotopic primary pancreatic cancer tumorgrafts (F2). The harvested human patient tumors from the NOD/SCID mice were transplanted

orthotopically into six-week-old transgenic GFP nude mice (F2 model). After 110 days, primary tumors were observed using the OV100 imaging system. The GFP stromal cells from the GFP host mouse had migrated into the orthotopic pancreatic tumor, causing the tumors to fluoresce bright green (22). Both GFP cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs) were observed in the primary tumor (Figure 1). Histological examination at 110 days of tumor growth revealed pancreatic tubular adenocarcinoma (Figure 1B).

GFP host stromal cells infiltrate peritoneal disseminated metastases of orthotopic pancreatic cancer tumorgrafts (F2). Fluorescent peritoneal metastases were examined with the OV100 imaging system. The GFP stromal cells from the GFP host mouse formed a capsule around the F2 disseminated peritoneal metastases (Figure 2A). Both GFP CAFs and TAMs were observed in the disseminated

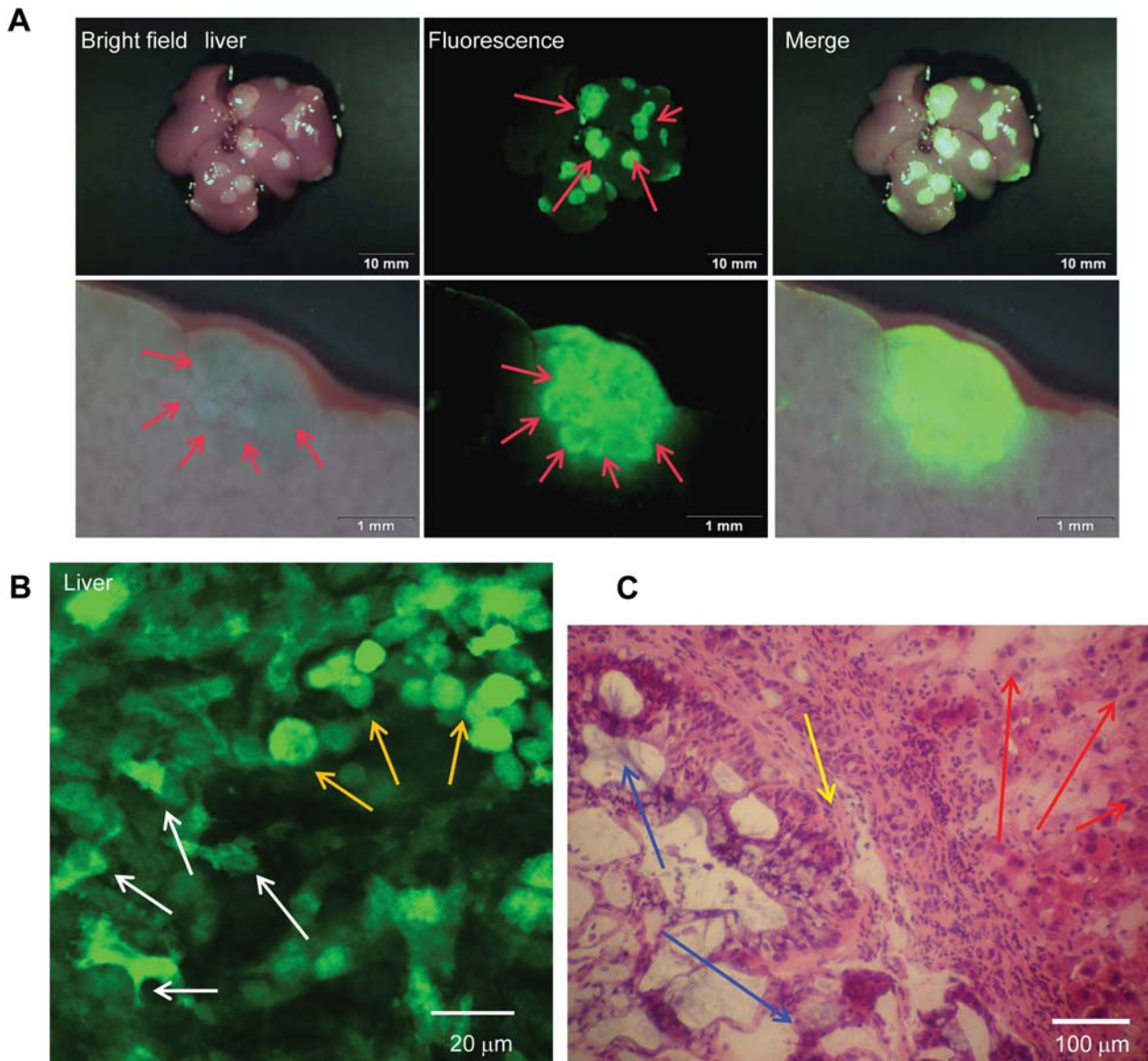


Figure 3. A: Upper image shows liver metastasis. Red arrows indicate liver metastasis with green fluorescent protein (GFP) stroma. Bar=10 mm. Lower panel shows high-magnification image of liver metastasis. Bar=1 mm. Red arrows indicate GFP stroma. B: Image of liver metastasis. Yellow arrows indicate tumor-associated macrophages (TAMs). White arrows indicate cancer-associated fibroblasts (CAFs). Image was taken with an Olympus FV1000 confocal laser microscope. Bar=20 μm. C: Liver metastasis stained with H&E. Blue arrows indicate pancreatic tubular adenocarcinoma. Yellow arrow indicate stromal cells. Red arrows indicate hepatocytes. Bar=100 μm.

peritoneal metastases (Figure 2B). Histological examination at 110 days of tumor growth revealed pancreatic tubular adenocarcinoma (Figure 2C), similar to the primary tumor.

GFP host stromal cells infiltrate liver metastases of orthotopic pancreatic cancer tumorgrafts (F2). On day 110 after orthotopic implantation of the patient pancreatic tumor, GFP fluorescence was observed in the experimental liver

metastases with the OV100 imaging system (Figure 3A). High-magnification fluorescence imaging showed extensive GFP fluorescence in the liver metastasis (Figure 3A). Host GFP cells extensively accumulated in the liver metastasis. Both GFP CAFs and TAMs were observed in the liver metastasis (Figure 3B). Histological examination of the liver metastasis revealed pancreatic tubular adenocarcinoma (Figure 3C).

Discussion

There are many advantages of studying low-passage mouse models of patient-tumor specimens. Most importantly, there is minimal deviation from the tumor as it was in the patient. Secondly, potentially individualized therapy for each patient's cancer can be tested on the tumor growing in a cohort of mice. Mouse models of individual cancer patients can therefore revolutionize cancer treatment. However, the proper mouse model should be used. The subcutaneous tumor models do not behave as the patient does (13-19). Firstly, there is no metastasis in the subcutaneous models, which is the lethal aspect of cancer that needs to be prevented or treated. Secondly, subcutaneous ectopic tumors behave in other ways very differently from orthotopic tumors. The ectopic tumor becomes encapsulated and is then basically benign and its drug response pattern can be altered with respect to tumors growing at an orthotopic site (26).

We have demonstrated in the current report a metastatic orthotopic mouse model of pancreatic patient tumors that become imageable due to stromal accumulation in the primary and metastatic tumors from the transgenic nude mouse host ubiquitously expressing GFP. The fluorescent-protein-expressing stroma included CAFs and TAMs. This patient-like model can be used to image primary tumor and metastatic progression of tumors from human patients to optimize individualized treatment of metastatic pancreatic and other cancer types and to discover novel therapeutics.

Conflict of Interest

None of the authors have a conflict of interest regarding this study.

Acknowledgements

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