Non-invasive Fluorescent-protein Imaging of Orthotopic Pancreatic-cancer-patient Tumorgraft Progression in Nude Mice

ATSUSHI SUETSUGU[1,2,4], MATTHEW KATZ[3], JASON FLEMING[3], MARK TRUTY[3], RYAN THOMAS[3], SHIGETOYO SAJI[4], HISATAKA MORIWAKI[4], MICHAEL BOUVET[2] and ROBERT M. HOFFMAN[1,2]

[1]*AntiCancer Inc. San Diego, CA, U.S.A.;*
[2]*Department of Surgery, University of California, San Diego, CA, U.S.A.;*
[3]*Department of Surgical Oncology, MD Anderson Cancer Center, Houston, TX, U.S.A.;*
[4]*Gifu University Graduate School of Medicine, Gifu, Japan*

Abstract. *In order to individualize and therefore have more effective treatment for pancreatic cancer, we have developed a multicolor, imageable, orthotopic mouse model for individual patients with pancreatic cancer by passaging their tumors through transgenic nude mice expressing green fluorescent protein (GFP) and red fluorescent protein (RFP). The tumors acquired brightly fluorescent stroma from the transgenic host mice, which was stably associated with the tumors through multiple passages. In the present study, pancreatic cancer patient tumor specimens were initially established in NOD.CB17-Prkdc^{scid}/NcrCrl (NOD/SCID) mice. The tumors were then passaged orthotopically into transgenic nude mice ubiquitously expressing GFP and subsequently to nude mice ubiquitously expressing RFP. The tumors, with very bright GFP and RFP stroma, were then orthotopically passaged to non-transgenic nude mice. It was possible to image the brightly fluorescent tumors non-invasively longitudinally as they progressed in the non-transgenic nude mice. This non-invasive imageable tumorgraft model will be valuable to screen for effective treatment options for individual patients with pancreatic cancer, as well as for the discovery of improved agents for this treatment-resistant disease.*

There has been much excitement recently about tumors from human patients implanted in immunodeficient mice. These models have now been given names such as "tumorgraft" (1) "xenopatients" (2). However, these new models are ectopic subcutaneous models, as were similar nude-mouse models of tumors from patients in the 1970s and 1980s (3-9), and, therefore, are not patient-like. Our laboratory pioneered on surgical orthotopic implantation (SOI) nude-mouse models from patient tumor specimens in the early 1990s (10, 11). These orthotopic models are much more patient-like than the ectopic subcutaneous models. However, in orthotopic models, it is difficult to visualize tumor growth and metastasis.

To address this problem of imaging such orthotopic tumorgrafts, we have recently developed the technology to introduce fluorescent protein-expressing stroma (12-18) into tumors by passaging tumorgrafts through transgenic nude mice expressing fluorescent proteins. Tumor specimens from patients with pancreatic cancer were initially established subcutaneously in NOD.CB17-*Prkd*^{cscid}/NcrCrl (NOD/SCID) mice immediately after surgery (19, 20). The tumors were then passaged in transgenic nude mice ubiquitously expressing red fluorescent protein (RFP), green fluorescent protein (GFP), or cyan fluorescent protein (CFP), whereby the tumor acquired RFP, GFP and CFP stroma, respectively. RFP, GFP and CFP stroma acquired by the tumors, including RFP- and GFP-expressing cancer-associated fibroblasts (CAFs), tumor-associated fibroblasts (TAMs) and blood vessels, persisted throughout at least three passages (21).

In subsequent experiments, the primary patient tumors, passaged in transgenic GFP-expressing nude mice, acquired GFP-expressing stroma and subsequently metastasized to the liver and also formed disseminated peritoneal metastases. The metastases maintained the GFP stroma from the primary tumor, and apparently acquired stroma from the metastatic site, resulting in their very bright fluorescence (22).

The present report describes the further development of tumors from patients with pancreatic cancer, that are brightly-labeled with fluorescent protein-expressing stroma, for non-invasive and longitudinal imaging in orthotopic nude mouse models.

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

Key Words: Human patients, pancreatic cancer, NOD/SCID mice, nude mice, GFP, RFP, transgenic nude mice, stroma, tumor microenvironment, non-invasive imaging.

Materials and Methods

GFP and RFP mice. Transgenic nude C57/B6-GFP- and RFP-expressing mice were obtained from AntiCancer, Inc. (San Diego, CA, USA). These transgenic nude mice express the fluorescent protein gene under the control of the chicken β-actin promoter and cytomegalovirus enhancer. Most of the tissues from these transgenic mice, with the exception of erythrocytes and hair, fluoresce under illumination with proper excitation light (14, 21).

Animal care. The transgenic nude mice were bred and maintained in a HEPA-filtered environment at AntiCancer Inc. with cages, food, water, and bedding sterilized by autoclaving. All surgical procedures and imaging were performed with the animals anesthetized by intramuscular injection of a ketamine mixture. All animal studies were conducted in accordance with the principles of and the procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals, under assurance number A3873-1.

Specimen collection. All patients provided informed consent and samples were procured and the initial establishment of patients tmors in NOD/SCID mice was conducted under the approval of the Institutional Review Board of the MD Anderson Cancer Center.

Tumor models.

Establishment of tumorgraft model (F1) of tumor from patients with pancreatic cancer: Tumor tissue from patients with pancreatic cancer obtained at surgery was cut into 3-mm^3 fragments and transplanted subcutaneously into NOD/SCID mice (19, 20).

Orthotopic tumorgraft (F2) of tumor from patients with pancreatic cancer in transgenic GFP-expressing nude mice: The F1 tumors from NOD/SCID mice were harvested and cut into 3-mm^3 fragments and then transplanted orthotopically (23) into six-week-old transgenic GFP-expressing nude mice (14) (F2 model).

Orthotopic tumorgraft (F3) of tumors from patients with pancreatic cancer in transgenic RFP-expressing nude mice: The F2 tumors from GFP mice were harvested and cut into 3-mm^3 fragments and then transplanted orthotopically (23) into six-week-old transgenic RFP-expressing nude mice (15) (F3 model). For each passage of F2-F3, the tumor grew for 70 days.

Orthotopic tumorgraft (F4) of pancreatic cancer of patients' tumors in non-transgenic nude mice: The F3 tumors were harvested from the RFP-expressing nude mice and cut into 3-mm^3 fragments then transplanted orthotopically (23) into six-week-old non-transgenic nude mice (F4 model).

Fluorescence imaging. The growing orthotopic tumor, with very bright GFP and RFP stroma, was fluorescently imaged non-invasively at days 21, 30 and 74 with the OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan).

Histological analysis. Tumors were excised and observed under confocal microscopy with the FV1000 (Olympus).

Results

Tumors from patients with pancreatic cancer were initially transplanted subcutaneously into NOD/SCID mice within two hours of surgery (Figure 1A) (19, 20). Tumors were detectable by day 30. The harvested human pancreatic cancer patient tumors from the NOD/SCID mice were transplanted orthotopically into six-week-old transgenic GFP-expressing nude mice (F2 model) where the tumor acquired GFP-expressing stroma. After 70 days, the F2 tumors were harvested from the GFP nude mice and were transplanted orthotopically into six-week-old transgenic nude RFP-expressing mice (F3 model), where the tumors acquired RFP-expressing stroma in addition to their GFP stroma. After 70 days, the F3 tumors were harvested from the RFP-expressing nude mice and cut into 3-mm^3 fragments and were transplanted orthotopically into six-week-old non-transgenic nude mice (F4 model). The growing orthotopic tumor maintained the very bright GFP and RFP stroma from previous passages.

Non-invasive imaging at days 21, 30 and 74 demonstrated extensive orthotopic growth of the pancreatic cancer tumorgraft on the nude mouse pancreas (Figure 1B).

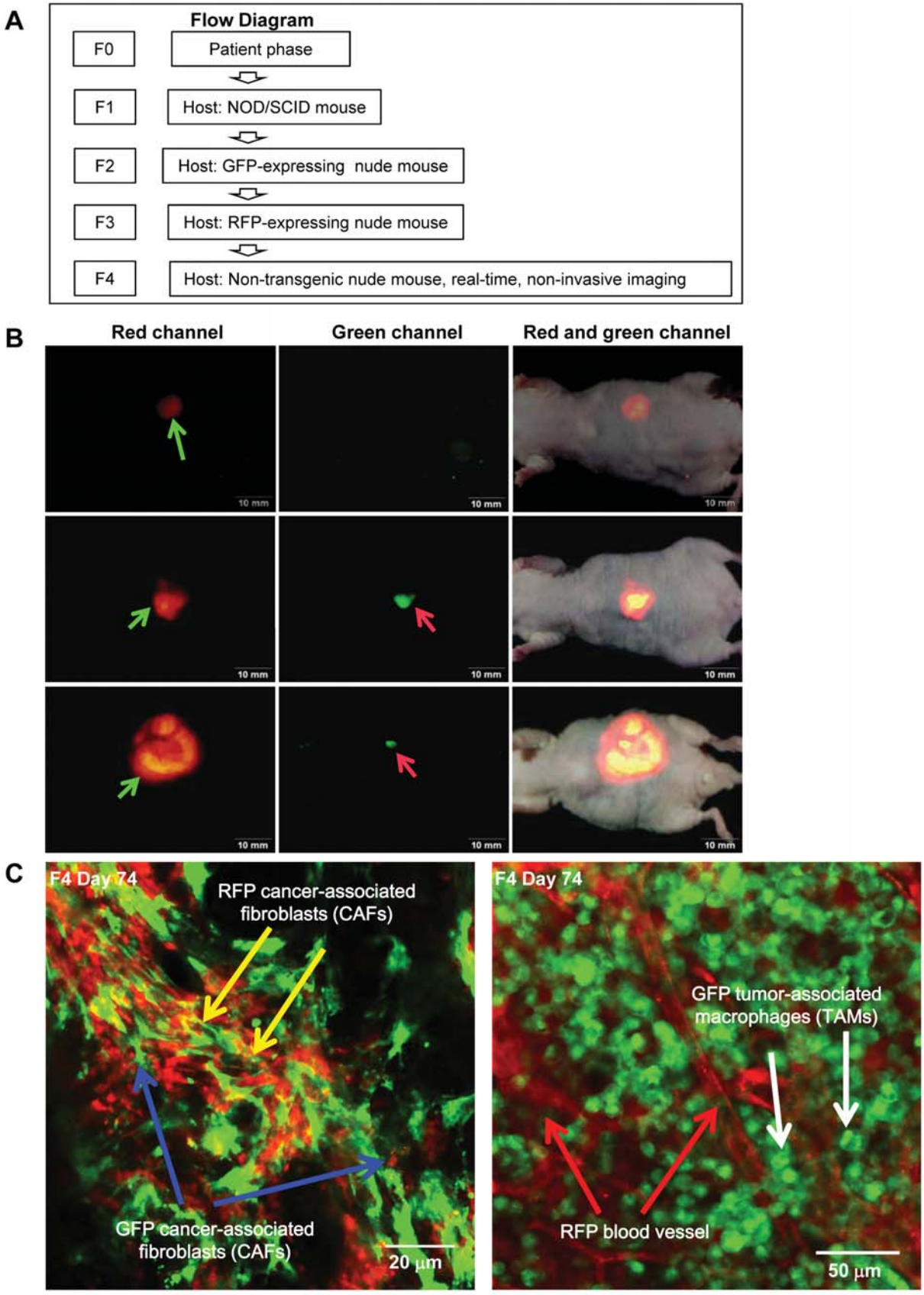
After resection, the F4 tumor was observed with the FV1000 confocal microscope (Figure 1C). RFP- and GFP-expressing cancer associated fibroblasts (CAFs), tumor associated macrophages (TAMs) and blood vessels from the GFP-expressing and RFP-expressing hosts still persisted in the human pancreatic tumor after the three passages described above (Figure 1C).

Discussion

We have demonstrated a new mouse model of patient-derived tumors whereby GFP- and RFP-expressing stromal elements are acquired by passaging the patients' pancreatic-cancer tumorgrafts through transgenic GFP- and RFP-expressing nude mice. The brightly fluorescent stroma enables the

→

Figure 1. *Non-invasive imaging of fluorescent tumor from (a) patient with pancreatic cancer growing orthotopically in nude mice. A: Flow diagram of the experimental protocol. B: Whole-body non-invasive imaging of human pancreatic cancer orthotopic tumorgraft in non-transgenic nude mice. Mice were non-invasively imaged at day-21 (upper row), day-30 (middle row) and day-74 (lower row). The tumors in the non-transgenic nude mice are in the F4 passage after F1, in NOD/SCID mice after patient surgery; F2, in transgenic green fluorescent protein (GFP)-expressing nude mice; and F3 in transgenic red fluorescent protein (RFP)-expressing nude mice. The tumor acquired GFP and RFP stroma in the F2 and F3 passages, respectively. Green arrows indicate tumor with RFP stroma. Red arrows indicate tumor with GFP stroma. Images were taken with the Olympus OV100 Small Animal Imaging System. C: Image of human pancreatic cancer tumor tissue resected from the F4 passage with RFP and GFP stroma. Images were taken with an FV1000 confocal laser microscope. Left panel, RFP-expressing and GFP-expressing cancer-associated fibroblast cells (CAFs). Right panel, RFP-expressing blood vessels and GFP-expressing tumor-associated macrophages (TAMs). Yellow arrows indicate RFP-expressing CAFs. Blue arrows indicate GFP-expressing CAFs. White arrows indicate GFP-expressing TAMs. Red arrows indicate RFP-expressing blood vessels.*


A
Flow Diagram
F0
Patient phase
F1
Host: NOD/SCID mouse
F2
Host: GFP-expressing nude mouse
F3
Host: RFP-expressing nude mouse
F4
Host: Non-transgenic nude mouse, real-time, non-invasive imaging
B
Red channel
Green channel
Red and green channel
10 mm
C
F4 Day 74
RFP cancer-associated fibroblasts (CAFs)
GFP cancer-associated fibroblasts (CAFs)
20 μm
F4 Day 74
GFP tumor-associated macrophages (TAMs)
RFP blood vessel
50 μm

patient-derived tumor to be non-invasively and longitudinally imaged after subsequent passage in non-transgenic nude mice. The fluorescent protein-expressing stroma included CAFs and TAMs. The fluorescent stroma persisted for at least three passages in the tumors growing in the transgenic and non-transgenic nude mice. The persistence of the stroma over multiple passages indicates the intimacy of cancer cells and stroma further demonstrates (24).

The new non-invasive imaging orthotopic model of cancer patient-derived tumors is superior to the ectopic, non-imageable models currently in use (10, 11, 25, 26).

This nude mouse model, described in this report, can be used to visualize primary and metastatic progression of human-derived tumors over a long period of time and their response to cancer therapy, as well as to stromal therapy. This model will allow standard and experimental drugs to be rapidly screened for patients which should individualize and improve therapy. The model described here will also improve our ability to discover novel effective agents for pancreatic and other cancer types.

Conflict of Interest

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

Acknowledgements

This study was supported in part by the National Cancer Institute grant CA132791.

References

1 Garber K: Personal mouse colonies give hope for pancreatic cancer patients. J Natl Cancer Inst *99*: 105-107, 2007.

2 Bertotti A, Migliardi G, Galimi F, Sassi F, Torti D, Isella C, Cora D, Di Nicolantonio F, Buscarino M, Petti C, Ribero D, Russolillo N, Muratore A, Massucco P, Pisacane A, Molinaro L, Valtorta E, Sartore-Bianchi A, Risio M, Capussotti L, Gambacorta M, Siena S, Medico E, Sapino A, Marsoni S, Comoglio PM, Bardelli A and Trusolino L: A molecularly annotated platform of patient-derived xenografts ("Xenopatients") identified HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. Cancer Disc *1*: 509-523, 2011.

3 Rygaard J and Povlsen CO: Heterotransplantation of a human malignant tumour to "Nude" mice. Acta Pathol Microbiol Scand *77*: 758-760, 1969.

4 Fiebig HH, Widmer K-H, Fiedler L, Wittekind C and Lohr GW: Development and characterization of 51 human tumor models for large bowel, stomach and esophageal cancer. Digestive Surgery *1*: 225-235, 1984.

5 Fiebig HH, Schmid JR, Bieser W, Henss H and Lohr GW: Colony assay with human tumor xenografts, murine tumors and human bone marrow. Potential for anticancer drug development. Eur J Cancer Clin Oncol *23*: 937-948, 1987.

6 Pickard RG, Cobb LM and Steel GG: The growth kinetics of xenografts of human colorectal tumours in immune deprived mice. Br J Cancer *31*: 36-45, 1975.

7 Selby PJ, Courtenay VD, McElwain TJ, Peckham MJ and Steel GG: Colony growth and clonogenic cell survival in human melanoma xenografts treated with chemotherapy. Br J Cancer *42*: 438-447, 1980.

8 Sharkey FE and Fogh J: Consideration in the use of nude mice for cancer research. Cancer Metastasis Rev 3: 341-360, 1984.

9 Giovanella BC, Stehlin JS Jr., Williams LJ Jr., Lee S-S and Shepard RC: Heterotransplantation of human cancers into nude mice. Cancer *42*: 2269-2281, 1978.

10 Fu X, Guadagni F and Hoffman RM: A metastatic nude-mouse model of human pancreatic cancer constructed orthotopically with histologically intact patient specimens. Proc Natl Acad Sci USA *89*: 5645-5649, 1992.

11 Fu X, Besterman JM, Monosov A and Hoffman RM: Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimens. Proc Natl Acad Sci USA *88*: 9345-9349, 1991.

12 McElroy M, Kaushal S, Bouvet M, Hoffman RM: Color-coded imaging of splenocyte pancreatic cancer cell interactions in the tumor microenvironment. Cell Cycle *7*: 2916-2921, 2008.

13 Yang M, Li L, Jiang P, Moossa AR, Penman S and Hoffman RM: Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and stromal cells. Proc Natl Acad Sci USA *100*: 14259-14262, 2003.

14 Yang M, Reynoso J, Jiang P, Li L, Moossa AR and Hoffman RM: Transgenic nude mouse with ubiquitous green fluorescent protein expression as a host for human tumors. Cancer Res *64*: 8651-8656, 2004.

15 Yang M, Reynoso J, Bouvet M, and Hoffman RM: A transgenic red fluorescent protein-expressing nude mouse for color-coded imaging of the tumor microenvironment. J Cell Biochem *106*: 279-284, 2009.

16 Tran Cao HS, Reynoso J, Yang M, Kimura H, Kaushal S, Snyder CS, Hoffman RM and Bouvet M: Development of the transgenic cyan fluorescent protein (CFP)-expressing nude mouse for "Technicolor" cancer imaging. J Cell Biochem *107*: 328-334, 2009.

17 Suetsugu A, Hassanein MK, Reynoso J, Osawa Y, Nagaki M, Moriwaki H, Saji S, Bouvet M and Hoffman RM: The cyan fluorescent protein nude mouse as a host for multicolor-coded imaging models of primary and metastatic tumor microenvironments. Anticancer Res *32*: 31-38, 2012

18 Hoffman RM and Yang M: Color-coded fluorescence imaging of tumor-host interactions. Nature Protoc *1*: 928-935, 2006

19 Kim MP, Evans DB, Wang H, Abbruzzese JL, Fleming JB and Gallick GE: Generation of orthotopic and heterotopic human pancreatic cancer xenografts in immunodeficient mice. Nat Protoc *4*: 1670-1680, 2009.

20 Kim MP, Truty MJ, Choi W, Kang Y, Chopin-Lally X, Gallick GE, Wang H, McConkey DJ, Hwang R, Logsdon C, Abbruzzesse J and Fleming JB: Molecular profiling of direct xenograft tumors established from human pancreatic adenocarcinoma after neoadjuvant therapy. Ann Surg Oncol, 2011 [Epub ahead of print] (DOI: 10.1245/s10434-011-1839-4).

21 Suetsugu A, Katz M, Fleming J, Truty M, Thomas R, Moriwaki H, Bouvet M, Saji S and Hoffman RM: Multi-color pallete of fluorescent proteins for imaging the tumor microenvironment of orthotopic tumorgraft mouse models of clinical pancreatic cancer specimens. J Cell Biochem *113*: 2290-2295, 2012.

22 Suetsugu A, Katz M, Fleming J, Truty M, Thomas R, Saji S, Moriwaki H, Bouvet M, Saji S and Hoffman RM: Imageable fluorescent metastasis resulting in transgenic GFP mice orthotopically implanted with human-patient primary pancreatic cancer specimens. Anticancer Res *32*: 1175-1180, 2012.
23 Hoffman RM: Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: A bridge to the clinic. Investig New Drugs *17*: 343-359, 1999.
24 Bouvet M, Tsuji K, Yang M, Jiang P, Moossa AR and Hoffman RM: *In vivo* color-coded imaging of the interaction of colon cancer cells and splenocytes in the formation of liver metastases. Cancer Res *66*: 11293-11297, 2006.
25 Rubio-Viqueira B, Jimeno A, Cusatis G, Zhang X, Iacobuzio-Donahue C, Karikari C, Shi C, Danenberg K, Danenberg PV, Kuramochi H, Tanaka K, Singh S, Salimi-Moosavi H, Bouraoud N, Amador ML, Altiok S, Kulesza P, Yeo C, Messersmith W, Eshleman J, Hruban RH, Maitra A and Hidalgo M: An *in vivo* platform for translational drug development in pancreatic cancer. Clin Cancer Res *12*: 4652-4661, 2006.
26 Embuscado EE, Laheru D, Ricci F, Yun KJ, de Boom Witzel S, Seigel A, Flickinger K, Hidalgo M, Bova GS and Iacobuzio-Donahue CA: Immortalizing the complexity of cancer metastasis: genetic features of lethal metastatic pancreatic cancer obtained from rapid autopsy. Cancer Biol Ther *4*: 548-554, 2005.

Received April 12, 2012
Revised June 9, 2012
Accepted June 11, 2012