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Efficacy of *Salmonella typhimurium* A1-R Versus Chemotherapy on a Pancreatic Cancer Patient-Derived Orthotopic Xenograft (PDOX)

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ABSTRACT

The aim of this study is to determine the efficacy of tumor-targeting *Salmonella typhimurium* A1-R (A1-R) on pancreatic cancer patient-derived orthotopic xenografts (PDOX). The PDOX model was originally established from a pancreatic cancer patient in SCID-NOD mice. The pancreatic cancer PDOX was subsequently transplanted by surgical orthotopic implantation (SOI) in transgenic nude red fluorescent protein (RFP) mice in order that the PDOX stably acquired red fluorescent protein (RFP)-expressing stroma for the purpose of imaging the tumor after passage to non-transgenic nude mice in order to visualize tumor growth and drug efficacy. The nude mice with human pancreatic PDOX were treated with A1-R or standard chemotherapy, including gemcitabine (GEM), which is first-line therapy for pancreatic cancer, for comparison of efficacy. A1-R treatment significantly reduced tumor weight, as well as tumor fluorescence area, compared to untreated control ($P = 0.011$), with comparable efficacy of GEM, CDDP, and 5-FU. Histopathological response to treatment was defined according to Evans's criteria and A1-R had increased efficacy compared to standard chemotherapy. The present report is the first to show that A1-R is effective against a very low-passage patient tumor, in this case, pancreatic cancer. The data of the present report suggest A1-1 will have clinical activity in pancreatic cancer, a highly lethal and treatment-resistant disease and may be most effectively used in combination with other agents. J. Cell. Biochem. 115: 1254–1261, 2014.

KEY WORDS: *Salmonella typhimurium*; PANCREATIC CANCER; ORTHOTOPIC; NUDE MICE; RFP; PDOX; FLUORESCENT PROTEINS; IMAGING

There is a long history of bacteria infection apparently inducing cancer patients to go into remission. For a time, in the late 19th century and early 20th century, bacteria or bacterial extracts were used to treat cancer patients [Nauts et al., 1953]. Recently, there has been great interest in bacterial therapy of cancer using *Salmonella typhimurium* Toso et al., 2002; Pawelek et al., 2003; Jia et al., 2007; Forbes, 2010; Flentie et al., 2012]. We have previously developed a genetically-modified bacteria strain,

Abbreviations: 5-FU, 5-fluorouracil; CDDP, cisplatin; GEM, gemcitabine; H&E, hematoxylin and eosin; PDOX, patient-derived orthotopic xenograft; RFP, red fluorescent protein; SOI, surgical orthotopic implantation.

The authors declare that they have no competing interests.

This paper is dedicated to the memory of A.R. Moossa, M.D.

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Salmonella typhimurium A1 (A1), selected for tumor targeting in vivo. A1 grew in tumors, but not normal tissue due to its attenuation by auxotrophy for leu and arg [Zhao et al., 2005]. In order to increase tumor-targeting capability of A1, the strain was re-isolated by passage through a tumor growing in nude mice. The tumor-isolated strain, termed *S. typhimurium* (A1-R), had increased targeting for cancer cells in vivo as well as in vitro compared with A1 [Zhao et al., 2005, 2006, 2007; Hoffman, 2012]. We have demonstrated the efficacy of A1-R treatment in various types of human and mouse cancer cell lines [Hayashi et al., 2009a,b; Kimura et al., 2010; Hoffman, 2012; Momiyama et al., 2012; Zhao et al., 2012] including pancreatic cancer [Nagakura et al., 2009; Yam et al., 2010] and pancreatic cancer stem-like cells [Hiroshima et al., 2013]. However, it is not known whether *S. typhimurium* A1-R is active against patient-tumor mouse models.

Orthotopic nude mouse models were the first to enable metastasis to occur in nude mice [Fidler, 1990]. However, the early orthotopic models utilized cell suspensions for orthotopic implantation, usually resulting in low frequencies of metastasis. The use of intact tissue fragments for surgical orthotopic implantation (SOI) greatly increased the metastatic frequency of tumors growing in nude mice [Fu et al., 1991, 1992; Fu and Hoffman, 1993; Furukawa et al., 1993a].

We previously compared SOI and orthotopic cell suspension implantation in a stomach cancer model. With SOI, 100% of the mice had metastasis to the regional lymph nodes, liver, and lung. In contrast, when cell suspensions were used to inject stomach cancer cells at the same site, metastases occurred in only 6.7% of the mice [Furukawa et al., 1993b].

The advantages of SOI over orthotopic cell suspension implantation was demonstrated to be a general phenomenon [Hoffman, 1999].

Mouse models of patient tumors are more related to the patient than high-passage cancer cell lines [Talmadge et al., 2007; Bertotti et al., 2011]. Patient-derived orthotopic xenograft (PDOX) mouse models replicate the natural course of the patient tumor [Fu et al., 1991, 1992, 1993a; Fu and Hoffman, 1993; Furukawa et al., 1993b] than subcutaneous transplant models of patient tumors in nude mice, which now have names such as "tumorgraft" [Gerber, 2009] or "Xenopatient" [Bertotti et al., 2011].

In the current report, we used the fluorescent PDOX (fPDOX) that we developed from a pancreatic cancer patient [Suetsugu et al., 2012a,b,c] by passage in a red fluorescent protein (RFP)-expressing nude mouse, thereby stably acquiring RFP stroma for imaging upon SOI transplantation to a non-transgenic nude mouse in order to compare the efficacy of *S. typhimurium* A1-R and standard chemotherapy. We show for the first time that *S. typhimurium* A1-R is active against a low-passage patient tumor, suggesting the clinical potential of bacterial therapy against this highly lethal type of cancer.

MATERIALS AND METHODS

SPECIMEN COLLECTION

All patients provided informed written consent, and samples were procured and initially implanted in SCID/NOD mice under the approval of the Institutional Review Board of the MD Anderson Cancer Center [Kim et al., 2009, 2012].

ANIMALS

Athymic (*nu*/*nu*) mice and transgenic nude ubiquitously-expressing red fluorescent protein (RFP) mice [Yang et al., 2009] (AntiCancer, Inc., San Diego, CA), all 4–6 weeks of age, were used. The transgenic nude mice express the RFP gene under the control of the chicken β-actin promoter and cytomegalovirus enhancer [Vintersten et al., 2004]. Mice were kept in a barrier facility under HEPA filtration. Mice were fed with an autoclaved laboratory rodent diet. 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 maleate (Butler-Schein, Dublin, OH). All animal studies were conducted with an Institutional Animal Care and Use Committee (IACUC)-protocol approved for this study and in accordance with the principals and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

ESTABLISHMENT OF PDOX MODEL

Pancreatic cancer tumor tissue from a single patient was originally obtained at surgery at the MD Anderson Cancer Center and cut into 3-mm^3 fragments and transplanted subcutaneously in NOD/SCID mice (F1 generation) [Kim et al., 2009, 2012].

ESTABLISHMENT OF fPDOX MODEL

The fPDOX model was established, using SOI, in transgenic RFP nude mice (F2) [Fu et al., 1992; Yang et al., 2009], from the patient tissue growing in NOD/SCID mice. A small 6- to 10-mm transverse incision was made on the left flank of the RFP nude mouse through the skin and peritoneum. The tail of the pancreas was exposed through this incision, and a single 1-mm^3 tumor fragment from the F1 tumor was sutured to the tail of the pancreas using 8-0 nylon surgical sutures (Ethilon; Ethicon, Inc., NJ). On completion, the tail of the pancreas was returned to the abdomen and the incision was closed in one layer using 6-0 nylon surgical sutures (Ethilon) [Fu et al., 1992; Hoffman, 1999; Bouvet et al., 2000; Bouvet et al., 2002; Katz et al., 2003]. This model was the F-2 generation.

The F2 tumors were harvested from transgenic nude RFP mice and passed orthotopically in non-transgenic nude mice using SOI.

TISSUE HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Tumor samples were removed with surrounding normal tissues at the time of resection. Fresh tissue samples were fixed in 10% formalin and embedded in paraffin before sectioning and staining. Tissue sections (3 μm) were deparaffinized in xylene and rehydrated in an ethanol series. For immunohistochemistry, the sections were then treated for 30 min with 0.3% hydrogen peroxide to block endogenous peroxidase activity. The sections were subsequently washed with PBS and unmasked in citrate antigen unmasking solution (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan) in a water bath for 40 min at 98 °C. After incubation with 10% normal goat serum, the sections were incubated with anti-human MHC class I (1:100; Abcam, Inc., MA) and anti-MHC class I H2 Kd + H2 Dd (1:100; Abcam, Inc.) at 4 °C overnight. The binding of primary antibodies

was detected using anti-rabbit and anti-mouse secondary antibodies and avidin/biotin/horseradish peroxidase complex (DAKO Cytomation, Kyoto, Japan) for 30 min at room temperature. The labeled antigens were visualized with the DAB kit (DAKO Cytomation). Finally, the sections were counterstained with hematoxylin and examined using a BH-2 microscope (Olympus Corp., Tokyo, Japan) equipped with an INFINITY1 2.0 megapixel CMOS digital camera (Lumenera Corporation, Ottawa, Canada). All images were acquired using INFINITY ANALYZE software (Lumenera Corporation) without post-acquisition processing.

FROZEN TISSUE SECTIONS

The F2 orthotopic tumor tissue, with RFP stroma, was embedded using optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek Europe BV, Zoeterwude, the Netherlands) and preserved in liquid nitrogen. The frozen tissue was sectioned at 7 μm with a Cryomicrotome (Leica CM1850, Wetzler, Germany).

IMAGING

Frozen sections were directly observed with confocal microscopy (Fluoview FV1000, Olympus Corp.). The excitation source was a laser emitting 559 nm for RFP. Fluorescence images were obtained using the 20×/1.0 XLUMPLFLN objective [Uchugonova et al., 2011]. Imaging of tumors was performed with the OV100 Small Animal Imaging System (Olympus Corp.) [Yamauchi et al., 2006].

PREPARATION OF BACTERIA

The *S. typhimurium* A1-R bacteria were grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-log phase, washed with PBS, and then diluted in PBS. Bacteria were then used for in vivo experiments [Zhao et al., 2006].

CHEMOTHERAPY

The mice were treated in the following groups: (1) 5-fluorouracil (5-FU): 10 mg/kg, i.p.; (2) cisplatin (CDDP): 5 mg/kg, i.p.; (3) gemcitabine (GEM): 150 mg/kg, i.p.; (4) *Salmonella typhimuium* A1-R: 1.5×10^8 CFU/body, i.p.; and (5) saline (vehicle/control): 150 μl, i.p. Chemotherapy drugs were administered weekly beginning from day 21 after transplantation for a subsequent 4 weeks. Each treatment arm involved 5 tumor-bearing mice. No significant effects on body weight, morbidity, or severe toxicity were observed in any treatment arms. Animals were sacrificed at 7 weeks, and tumors were imaged using the OV100. At necropsy, tumors were weighed and harvested for analysis. The tumor volume was estimated by measuring the fluorescent area with the National

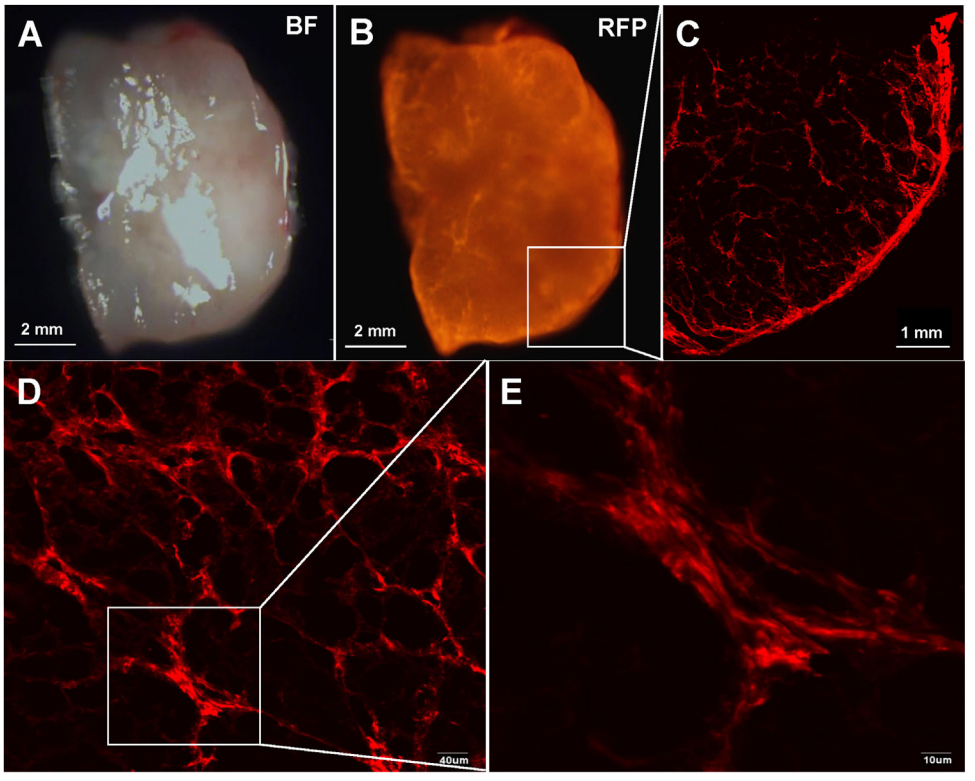

Fig. 1. Stroma of fPDOX (F2) implanted in RFP transgenic nude mice. The F2 fPDOX was excised from an RFP nude mouse. Cross-section images with bright field (A) and fluorescence (B) (OV100 Small Animal Imaging System, Olympus Corp.). (C) Confocal imaging of the PDOX with the FV1000 (Olympus Corp.). The RFP stromal cells from the RFP host mice formed a capsule around the F2 tumor and infiltrated into the central part of the tumor (D). RFP-expressing fibroblasts could be visualized in the tumor (E). Scale bars: (A) and (B), 2 mm; (C), 1 mm; (D), 40 μm; (E), 10 μm. See Materials and Methods Section for details.

Institutes of Health (NIH) Image J analysis software program (available at http://rsb.info.nih.gov/nih-image).

EVALUATION OF HISTOPATHOLOGICAL RESPONSE TO CHEMOTHERAPY DRUGS

Histopathological response to chemotherapy drugs was defined according to Evans's grading scheme [Evans et al., 1992]. Paraffin-embedded sections (3 μm) from all primary tumors were stained with hematoxylin and eosin (H&E). Grade I, little (<10%) or no cancer cell destruction is evident; Grade II a, destruction of 10–50% of cancer cells; Grade II b, destruction of 51–90% of tumor cells; Grade III, few (<10%) viable-appearing cancer cells are present; Grade IV, no viable cancer cells are present. Two investigators, with no knowledge of the chemotherapy outcome, independently assessed the H&E stained sections. The results were based on the mean values of five visual fields at 10× magnification.

DATA PROCESSING AND STATISTICAL ANALYSIS

PASW Statistics 18.0 (SPSS, Inc.) was used for all statistical analyses. The Student's *t*-test was used to compare continuous variables between two groups. Analysis of variance models were used to compare multiple groups. Comparisons between categorical variables were analyzed using the Fisher exact test. *P* value of 0.05 was considered statistically significant for all comparisons. Spearman correlation coefficient and linear regression were used to assess the various possible relationships among different variables.

RESULTS

IDENTIFICATION OF MOUSE STROMAL CELLS AND HUMAN CANCER CELLS IN PDOX GROWN IN RFP TRANSGENIC NUDE MICE

The patient pancreatic cancer tumorgrafts harvested from the NOD/SCID mice were transplanted orthotopically in 6-week-old transgenic RFP nude mice by SOI. After 30 days, tumors were imaged using the OV100 (Fig. 1A,B). The RFP stromal cells from the RFP host mice formed a capsule around the F2 tumor (Fig. 1C) and infiltrated into the central part of the tumor (Fig. 1D). RFP-expressing fibroblasts could be visualized in the tumor (Fig. 1E). The stromal cells in the PDOX models reacted with anti-mouse MHC class I antibody but did not react with anti-human MHC class I antibody (Fig. 2), suggesting the stromal cells in the PDOX tumor consisted of host mouse cells. In contrast, the cancer cells strongly reacted with human HLA antibody indicating they were of human origin.

S. TYPHIMURIUM A1-R EFFICACY ON PANCREATIC CANCER PDOX COMPARED TO STANDARD CHEMOTHERAPY

The F2 tumors were harvested from transgenic nude RFP mice and passed orthotopically in non-transgenic nude mice by SOI. The nude mice were treated in the following groups: (1) 5-FU (10 mg/kg, i.p.); (2) CDDP (10 mg/kg, i.p.); (3) GEM (150 mg/kg, i.p.); (4) A1-R (1.5×10^8 CFU/body, i.p.) and (5) saline (vehicle/control) (i.p.). Each treatment arm involved 5 tumor-bearing mice. No significant effects on body weight, morbidity, or severe toxicity were observed in any

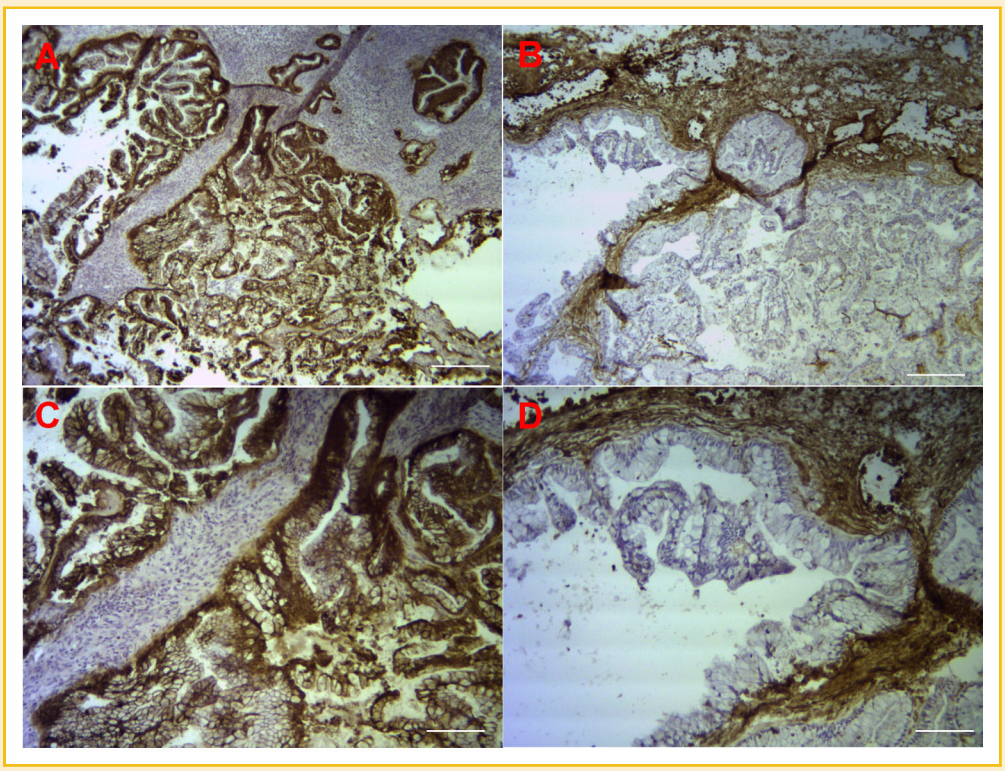

Fig. 2. Immunohistochemical characterization of PDOX cancer cells and stroma after growth in nude mice. Immunohistochemistry for human MHC class I (HLA) (A, C) and mouse MHC class I (H2 Kd + H2 Dd) (B,D). Strong staining of HLA was observed in the cancer cells (A,C), whereas strong staining of H2 Kd + H2 Dd was observed in the stromal cells. C,D: High magnification images (20×) of (A) and (B) respectively. Scale bars: (A) and (B), 250 μm; (C) and (D), 100 μm. See Materials and Methods Section for details.

treatment arm. After 4 weekly treatments and an additional 3 weeks, mice were sacrificed. The average tumor weight of each group was as follows: (1) 5-FU: 0.044 ± 0.027 g; (2) CDDP: 0.04 ± 0.032 g; (3) GEM: 0.058 ± 0.051 g; (4) A1-R: 0.106 ± 0.038 g; and (5) control: 0.258 ± 0.209 g. A1-R significantly reduced tumor weight compared to control treatment ($P = 0.011$), as did the other treatments including 5-FU ($P = 0.005$); CDDP ($P = 0.004$); and GEM ($P = 0.001$; Fig. 3B).

The fluorescent stroma growing in the patient pancreatic cancer tumorgraft was imaged with the OV100 in order to measure tumor fluorescence area and then estimate tumor volume (Fig. 3A). The mean fluorescence area of each group was as follows: (1) 5-FU: $1066.4 \pm 289.4\ mm^2$; (2) CDDP: $708.6 \pm 394.8\ mm^2$; (3) GEM: $1143.2 \pm 524.1\ mm^2$; (4) A1-R: $1458.8 \pm 604.8\ mm^2$; and (5) control: $5081.6 \pm 2356.4\ mm^2$ ($P = 0.011$) (Fig. 3C). Thus, both A1-R and standard chemotherapy significantly inhibited the growth of the pancreatic cancer PDOX.

Tumor weight and fluorescence area showed a strong positive correlation ($r = 0.948$; $P < 0.001$; Fig. 3D). Earlier studies had shown that tumor volume and fluorescence area had a strong-positive correlation [Katz et al., 2003].

EVAN'S GRADING SHOWED *S. TYPHIMURIUM* A1-R TO BE MOST EFFECTIVE

Histopathological response to treatment was defined according to Evans's grading criteria [Evans et al., 1992]. In control sections, the tumor was largely occupied by viable cancer cells (Fig. 4E). Approximately 30% of cancer cells were destroyed and replaced

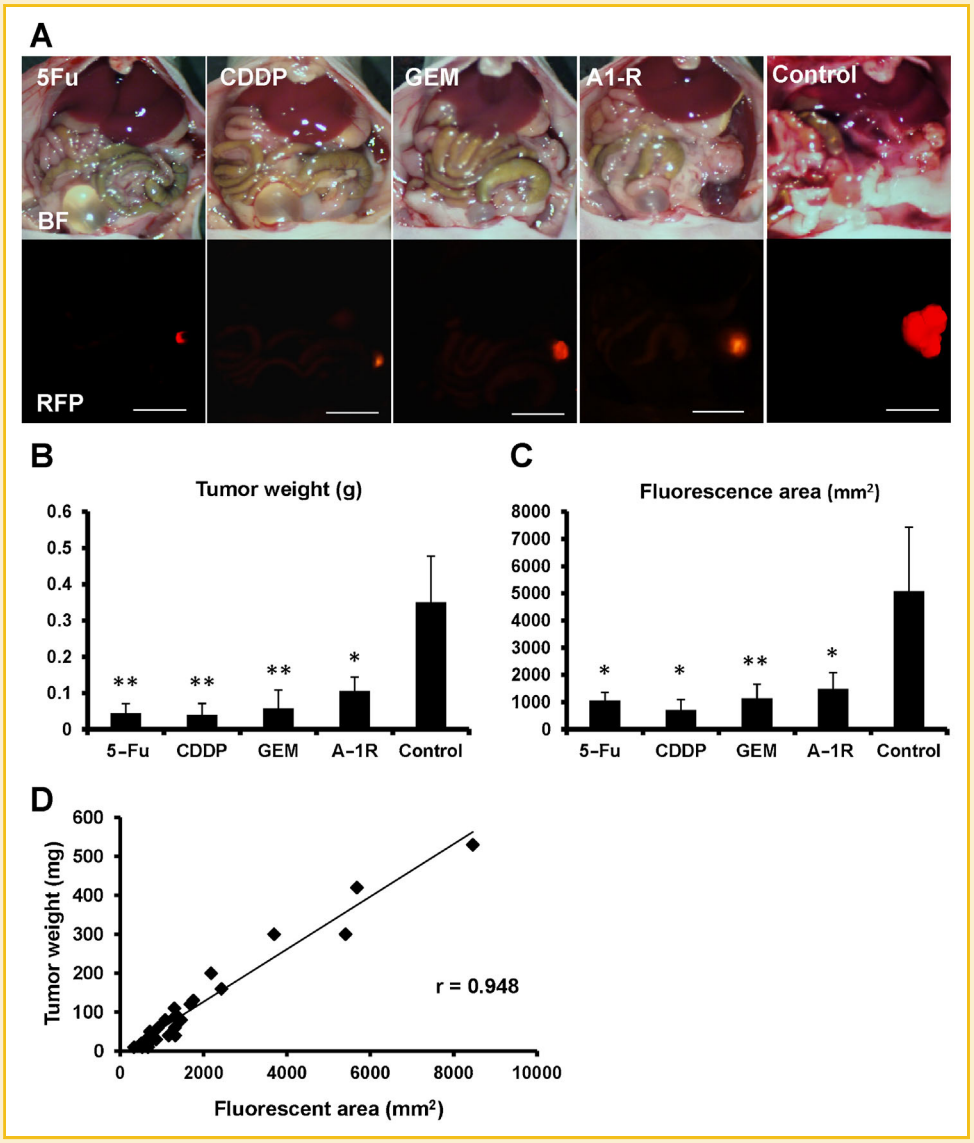

Fig. 3. Efficacy of *S. typhimurium* A1-R and standard chemotherapy on PDOX. A: Intravital imaging of tumor-bearing mice at termination. B: All treatments significantly reduced tumor weight compared to control (5-FU: $P = 0.005$; CDDP: $P = 0.004$; GEM: $P = 0.001$; A1-R: $P = 0.011$). C: All treatments significantly reduced fluorescent area compared to control (5-FU: $P = 0.018$; CDDP: $P = 0.013$; GEM: $P = 0.007$; A1-R: $P = 0.011$). D: Tumor weight and fluorescent area showed a strong positive correlation ($r = 0.948$; $P < 0.001$). Scale bars: 10 mm. *$P < 0.05$, **$P < 0.01$ (compared to control).

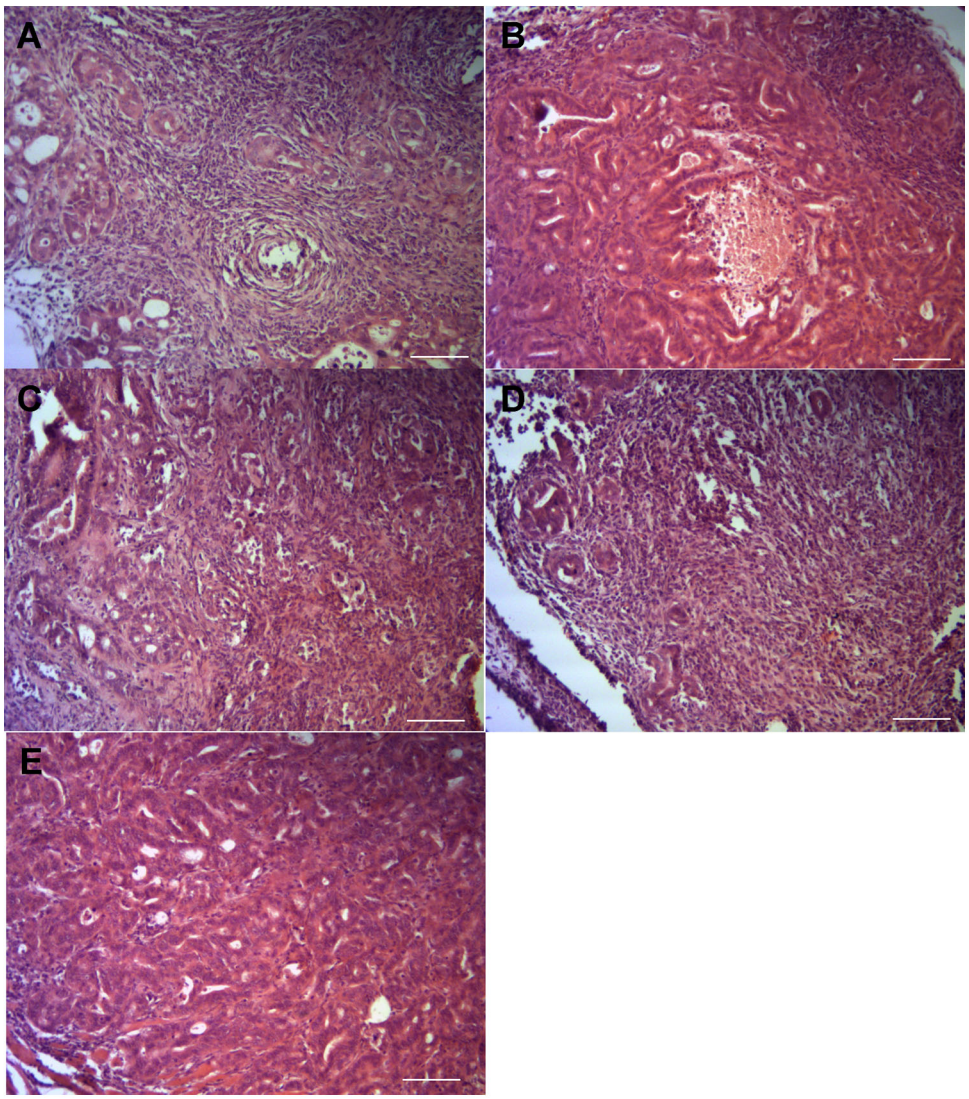

Fig. 4. Evaluation of histopathological response of PDOX to treatments using Evan's criteria. Histopathological response to treatment was defined according to Evans's grading scheme. A: 5-FU was judged as grade IIA; (B) CDDP: as IIB; (C) GEM: as IIB; and (D) A1-R: as III compared to (E) control. Scale bars: 100 μm.

by stromal cells in the tumor sections in animals treated with 5-FU (Fig. 4A); and 70% of cancer cells were destroyed in the tumor sections from animals treated with CDDP (Fig. 4B) or GEM (Fig. 4C). In contrast, only a few (<10%) viable cancer cells remained in the tumor sections treated with A1-R (Fig. 4D). The treatment efficacy of 5-FU was judged as grade IIA; CDDP as IIB; GEM as IIB; and A1-R as III, suggesting A1-R was histopathologically the most effective of all agents tested. *S. typhimurium* A1-R may have caused a strong fibrotic reaction in the PDOX tumor.

DISCUSSION

The present study used the PDOX model [Fu et al., 1991, 1992, 1993; Fu and Hoffman, 1993; Furukawa et al., 1993] of a patient pancreatic tumor with fluorescent stroma fPDOX [Suetsugu et al., 2012a,b,c] stably acquired by passage in transgenic RFP-nude mice. The pancreatic cancer fPDOX was then orthotopically transplanted to non-transgenic nude mice. The growth of the pancreatic cancer PDOX in the transgenic RFP nude mice enabled the PDOX to acquire RFP stroma and be subsequently imaged intravitally in non-transgenic nude mice in order to visualize tumor growth and drug efficacy.

The PDOX models were treated with A1-R or standard chemotherapy including gemcitabine (GEM), which is first-line treatment of pancreatic cancer in the clinic. A1-R treatment significantly reduced tumor weight as well as tumor fluorescent area, compared to untreated control ($P = 0.011$). The efficacy of *S. typhimurium* A1-R and chemotherapy, when determined by tumor weight and fluorescence area, are statistically similar indicating that *S. Typhimurium* A1-R is active against a patient tumor.

The main purpose of the present study was to demonstrate that the tumor-targeting strain *S. typhimurium* A1-R had comparable efficacy with chemotherapy. Most importantly, this study was done with a patient-derived orthotopic xenograft (PDOX), which is more clinically relevant than a cell line. This is the first study of *S. typhimurium* A1-R in a PDOX model. The results suggest the clinical potential of *S. typhimurium* A1-R since it has significant efficacy in a PDOX model of pancreatic cancer, a highly lethal disease. The present study also suggests that future studies include *S. typhimurium* A1-R in combination chemotherapy studies, preclinically and then clinically for pancreatic cancer.

The fluorescence area showed a strong positive correlation with tumor weight ($r = 0.948$; $P < 0.001$; Fig. 3D). It is also possible to image fPDOX non-invasively [Suetsugu et al., 2012c]. Thus, non-invasive imageable PDOX models 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.

Evans et al. (1992) grading scheme for determining treatment efficacy was used in the present study. Using Evan's criteria, A1-R was judged more effective than standard chemotherapy tested (Fig. 4). A1-R may have caused a strong fibrotic reaction in the PDOX tumor. This finding suggests that the histopathological assessment is also needed to assess treatment efficacy in addition to measuring tumor volume and weight.

The data in Figure 4 demonstrate significant cancer cell death of the tumor after *S. typhimurium* A1-R treatment. The present study used a single PDOX model and demonstrates the proof-of-principle that a patient pancreatic tumor is sensitive to bacterial therapy comparable to standard chemotherapy, and possibly, more sensitive. Future studies will test more patients' tumors in PDOX® models with more extensive cell death experiments.

Future studies will also focus on i.v. treatment with *S. typhimurium* A1-R on PDOX models of pancreatic cancer and determining the extent of tumor targeting by comparison of bacterial number in the tumor and other organs.

Although A1-R has been previously shown to be effective for pancreatic cancer cell lines and cell lines of other cancers, the present report is the first to show that A1-R is effective against a very low passage patient tumor, in this case, pancreatic cancer PDOX in nude mice. This is the first investigation on the use of *S. typhimurium* A1-R on a PDOX model, which has more clinical relevancy than the cell lines previously tested.

The data of the present report suggest A1-R will have clinical activity in pancreatic cancer, currently a highly treatment-resistant and lethal disease. Future studies will compare survival efficacy of A1-R both as monotherapy and in combination therapy with currently-used agents.

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