Dual Combination Therapy Targeting DR5 and EMMPRIN in Pancreatic Adenocarcinoma

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Abstract

The goal of the study was to assess the efficacy of combined extracellular matrix metalloprotease inducer (EMMPRIN)- and death receptor 5 (DR5)-targeted therapy for pancreatic adenocarcinoma in orthotopic mouse models with multimodal imaging. Cytotoxicity of anti-EMMPRIN antibody and anti-DR5 antibody (TRA-8) in MIA PaCa-2 and PANC-1 cell lines was measured by ATPlite assay in vitro. The distributions of Cy5.5-labeled TRA-8 and Cy3-labeled anti-EMMPRIN antibody in the 2 cell lines were analyzed by fluorescence imaging in vitro. Groups 1 to 12 of severe combined immunodeficient mice bearing orthotopic MIA PaCa-2 (groups 1–8) or PANC-1 (groups 9–12) tumors were used for in vivo studies. Dynamic contrast-enhanced–MRI was applied in group 1 (untreated) or group 2 (anti-EMMPRIN antibody). The tumor uptake of Tc-99m-labeled TRA-8 was measured in group 3 (untreated) and group 4 (anti-EMMPRIN antibody). Positron emission tomography/computed tomography imaging with 18F-FDG was applied in groups 5 to 12. Groups 5 to 8 (or groups 9 to 12) were untreated or treated with anti-EMMPRIN antibody, TRA-8, and combination, respectively. TRA-8 showed high killing efficacy for both MIA PaCa-2 and PANC-1 cells in vitro, but additional anti-EMMPRIN treatment did not improve the cytotoxicity. Cy5.5–TRA-8 formed cellular caps in both the cell lines, whereas the maximum signal intensity was correlated with TRA-8 cytotoxicity. Anti-EMMPRIN therapy significantly enhanced the tumor delivery of the MR contrast agent, but not Tc-99m–TRA-8. Tumor growth was significantly suppressed by the combination therapy, and the additive effect of the combination was shown in both MIA PaCa-2 and PANC-1 tumor models. Mol Cancer Ther; 11(2); 405–15. ©2011 AACR.

Introduction

Pancreatic cancer is a highly malignant disease and the fourth leading cause of cancer death in the United States (1). Due to the nonspecific symptoms, pancreatic cancer is typically detected at the very late stages (2), and therefore only 15% of patients are eligible for curable operation at diagnosis (3). Gemcitabine is the first-line therapeutic agent for unresectable pancreatic cancer, but offers only modest benefit (4). Radiation or erlotinib (a small-molecule targeting epidermal growth factor receptor) combined with gemcitabine delivered better efficacy than gemcitabine alone (5, 6), but the routine clinical application is not recommended because of the minimal benefit. More recently, Conroy and colleagues reported that FOLFIRINOX (quadruple therapy with oxaliplatin, irinotecan, leucovorin, and fluorouracil) extended the patient life significantly, but the median survival time was still less than a year (7).

A monomeric monoclonal antibody, TRA-8, specifically targets only death receptor 5 (DR5) among 5 TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) receptors (8). TRA-8 has been considered as a promising novel drug for pancreatic cancer (9, 10). Because DR5 is present in most cancer cells, but limited in normal cells, TRA-8 enables selective killing of cancer cells without causing severe side effects. TRA-8 induces DR5 aggregation triggering apoptosis (11) and suppressing cell proliferation (12). Because pancreatic cancer stem cells express higher level of DR5, TRA-8 will be able to suppress pancreatic tumor regrowth efficiently (13). The phase I clinical trial of the humanized TRA-8, tigatuzumab, was completed, and no adverse side effects were identified (14).

A monomeric monoclonal antibody targeting extracellular matrix metalloprotease inducer (EMMPRIN) was recently developed, and a significant anticancer effect was shown in orthotopic pancreatic cancer murine models (15). EMMPRIN is a membrane-bound glycoprotein expressed in pancreatic cancer with high incidence (16). Matrix metalloproteinases, stimulated by EMMPRIN, are essential to degrade extracellular matrix components and thereby to invade tissue boundaries (17–20). EMMPRIN also
affects tumor neovascularization by stimulating VEGF isoforms and VEGFR-2 (21), and therefore anti-EMMPRIN therapy is capable of suppressing tumor angiogenesis as well as cancer cell invasion and metastasis. The antiangiogenic effect may induce the normalization of tumor microvasculature, reducing interstitial pressure and thereby improving drug delivery, which may lead to a better treatment (22). In fact, we recently showed that anti-EMMPRIN therapy induced a synergy when used with gemcitabine in a pancreatic cancer model (23).

Antibody-based therapies for cancer are attractive because of minimal systemic toxicity compared with chemotherapy. Because a therapeutic antibody is specific for a target in 1 pathway, there is the potential for combining antibody therapies for additive or synergistic benefits. This study targeted both DR5 and EMMPRIN to maximize the overall therapeutic effect by directly inducing cancer cell apoptosis via the TRA-8 antibody while simultaneously suppressing tumor invasion, metastasis, and angiogenesis via the anti-EMMPRIN antibody. The efficacy of the combination approach was followed over time using multimodal imaging.

Materials and Methods

Reagents and cell lines

All reagents were from Fisher unless otherwise specified. Dr. Tong Zhou (University of Alabama at Birmingham, Birmingham, AL) provided purified monomeric monoclonal anti-EMMPRIN antibody (mouse origin IgG1 κ) and TRA-8. Cy5.5 and Cy3 were purchased from GE Healthcare Inc. Fresh Tc-99m pertechnetate was purchased from Birmingham Nuclear Pharmacy. 18F-FDG was purchased from PETNET Solutions. Two human pancreatic cell lines, MIA PaCa-2 and PANC-1, were obtained from Dr. Donald Buchsbaum (University of Alabama at Birmingham, Birmingham, AL) more than 6 months ago and have not tested for authentication in our laboratory. DR5 and EMMPRIN expressions in both MIA PaCa-2 and PANC-1 cells were validated by immunoblot analysis (24, 25). MIA PaCa-2 and PANC-1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Mediatech Inc.) supplemented with 10% fetal bovine serum (Hyclone). OmnipaqueTM Eagle’s Medium (DMEM; Mediatech Inc.) supplemented with 10% fetal bovine serum (Hyclone). OmniqueTM (iohexol, 350 mg/mL; GE Healthcare Inc.) and prohance (gadoteridol, an MR contrast agent; Bracco Diagnostics Inc.) were purchased from the University of Alabama at Birmingham Hospital Pharmacy.

Cell viability assay

In vitro viability assays for MIA PaCa-2 and PANC-1 cells were conducted with TRA-8 alone or in combination with anti-EMMPRIN antibody. For each cell line, a total of 1,000 cells were added to each well of 96-well plates (4 columns × 18 rows). TRA-8 was diluted to 4 different concentrations (0, 10, 50, and 500 ng/mL) and was added to 18 wells per TRA-8 concentration (same concentration at each column). Anti-EMMPRIN antibody was diluted to 3 different concentrations (0, 50, and 100 ng/mL) and was added to the 6 rows (24 wells) of cells per anti-EMMPRIN concentration. After 24 hours of incubation at 37°C in 5% CO2, the ATP level was determined by the ATPlite assay (Perkin-Elmer). The light emission from the wells of the plates was measured with an IVIS-100 imaging system (Caliper Life Sciences) and quantified by the vendor software. The luminescent exposure time was 60 seconds, whereas binning was 8 seconds. Regions of interest (ROI) were drawn manually around the area of each individual well in the well plate, and the intensity of light emitted from each ROI was measured. Data were normalized to light emission of an equal number of untreated cells otherwise incubated under the same conditions as the treated cells.

HYNIC conjugation and radiolabeling

HYNIC conjugation and radiolabeling were conducted for a biodistribution study. A fresh 1.8 mmol/L solution of succinimidyl 6-hydrazinonicoinate (HYNIC; courtesy of Dr. Gary Bridger, AnorMED Inc., Langley, British Columbia) in dimethylformamide was prepared. Forty picomoles was transferred to glass vials, followed by freezing at −90°C, then the solutions were vacuum dried with Advantage Benchtop Freeze Dryer (Virtis Co. Inc.) with the shelf temperature at −75°C and trap at −90°C. A vial was reconstituted with 1.0 mL of sodium phosphate buffer [0.15 mol/L (pH 7.80)] containing 1 mg of TRA-8 (HYNIC/antibody molar ratio = 6). After a 3-hour incubation at room temperature, the mixture was transferred to Slide-A-Lyzer dialysis cassette having molecular weight cutoff of 10,000 (Pierce) and then immersed into 1,000 mL of PBS (pH 7.4) overnight at 4°C. The HYNIC-modified antibody was labeled with Tc-99m with SnCl2/tricine as the transfer ligand (26), and the unbound Tc-99m was removed by G-25 Sephadex size exclusion chromatography. The protein concentrations of the collected fractions were measured by Lowry assay (27). The Tc-99m-labeled TRA-8 was more than 95%, as measured by thin-layer chromatography using separate strips developed with saline and methyl ethyl ketone.

Fluorescence imaging

Fluorescence imaging was carried out to analyze the cellular distributions of the antibodies. Monofunctional dyes Cy5.5 or Cy3 were conjugated to TRA-8 or anti-EMMPRIN monoclonal antibody, respectively, at a 3:1 molar ratio according to manufacturer’s instructions. Each of MIA PaCa-2 and PANC-1 cell lines (1 × 105 cells per plate) was seeded onto 35-mm glass bottom culture dishes (MatTek Corp.) and incubated in complete cell culture medium. At 16 hours after seeding, the cells were washed with PBS and then treated with Cy5.5–TRA-8 (5 μg/mL), Cy3–CD147 (5 μg/mL), a combination of the two, or control. At 24 hours posttreatment, the cells were washed with PBS and then 3 images (×20) were taken per each treatment with a Leica DMIRE2 inverted microscope equipped with a Nuance camera (CRI Inc.) for randomly selected areas.

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To quantify the distributions of Cy5.5–TRA-8 and Cy3–anti-EMMPRIN antibody, a line was drawn on a randomly selected cell (n = 10 per Image), and the signal intensities of the fluorophores located on the line were measured. The maximum and minimum signal values together with variance per size were determined on each cell line when the 2 fluorophore-labeled antibodies were used individually or combined. Variance per size (var/size) was calculated by

\[
\text{Var/Size} = \frac{\sum_{i=1}^{N} \left( \frac{\text{Sig}_i - \text{<Sig>}}{\text{PDF}_i} \right)^2 \times \text{PDF}_i}{N},
\]

where Sig\(_i\) is the signal intensity on the pixel \(i\), \(<\text{Sig}>\) is the mean signal intensity of all pixels on the line, PDF\(_i\) is the probability density function on the pixel \(i\), and \(N\) is the total number of pixels on the line. PDF\(_i\) was calculated by

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\text{PDF}_i = \frac{\text{Sig}_i / \text{Sig}_{\text{max}}}{\sum_{i=1}^{N} (\text{Sig}_i / \text{Sig}_{\text{max}})},
\]

where Sig\(_{\text{max}}\) is the maximum signal intensity on the line. Variance per size presents how randomly the fluorophore-labeled antibodies are distributed on the cell membrane and is smaller when the signal intensity is more focused on a restricted region. The signal intensities of fluorophores were measured by ImageJ (version 1.40g; NIH).

Animal modeling

Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham. Twelve groups of female severe combined immunodeficient BALB/c mice (NCI-Frederick Animal Production Program, Frederick, MD, 4 ~ 6 weeks old, \(n = 5\) for groups 1–4 and \(n = 6\) for groups 5–12) were used. The procedure for intrapancreatic tumor implantation was the following: a 1 cm incision was made in the left upper quadrant of the abdomen of anesthetized mice, and a solution of 2.5 × 10\(^8\) MIA PaCa-2 (groups 5–8) or PANC-1 (groups 9–12) cells in 30 \(\mu\)L of DMEM was injected into the tail of the pancreas. The skin and peritoneum were closed in 1 layer with 3 interrupted 5–0 Prolene sutures. Drug dosing started at 21 days after cell implantation for groups 5 to 8, or 28 days after cell implantation for groups 9 to 12, to model established pancreatic tumors for all groups. For groups 1 and 2, a vascular access port (PennyPort, Access Technologies) was subcutaneously implanted on the right shoulder of each mouse and the catheter connected to the port was inserted into a jugular vein to facilitate repeated intravenous gadoxidoterol injections, at 4 days before dosing (day 17), as described in our previous studies (28, 29). Groups 1 and 2 were intraperitoneally injected with PBS (serving as control) and anti-EMMPRIN antibody (0.2 mg) respectively on day 21, and dynamic contrast-enhanced (DCE)-MRI was applied on days 21 (prior to dosing; baseline imaging) and 24. Groups 3 and 4 were intraperitoneally injected with PBS (serving as control) and anti-EMMPRIN antibody (0.2 mg), respectively, twice for a week. At 1 week after therapy initiation, groups 3 and 4 were intravenously injected with Tc-99m–TRA-8 (0.40 ± 0.02 MBq; 2.6 ± 0.1 \(\mu\)g), and biodistribution study was followed at 24 hours thereafter. Groups 5 to 8 were intraperitoneally injected with PBS (serving as control), anti-EMMPRIN antibody (0.2 mg, semiweekly), TRA-8 (0.1 mg, weekly), and combination, respectively, for 2 weeks, whereas \(^{18}\)F-FDG positron emission tomography/computed tomography (PET/CT) imaging was applied weekly. Same dosing and imaging schedules were applied for groups 9 to 12. All tumors of groups 5 to 12 were collected for Ki67 and CD31 staining at the end of the imaging study. All animals were anesthetized with isoflurane gas (1%~2%) during imaging.

MR imaging

Small animal MRI was conducted with a Bruker BioSpec 9.4T system (Bruker BioSpin Corp.) to assess the changes of microvasculature. The tumor was imaged with a combination of a \(^1\)H volume resonator/transmitter and a surface coil receiver (Bruker BioSpin Corp.). Respiratory rate of animals was monitored with an MR compatible small animal respiratory monitoring device (SA Instrument, Inc.) during imaging. A 27-gauge needle connected to a sterilized polyurethane tube (Strategic Applications Inc.) was inserted into the lumen of each port, to deliver gadoxidoterol. Anatomic MRI to measure tumor volume was carried out with a relaxation enhancement T2-weighted spin-echo sequence with the following acquisition parameters: repetition time (TR)/echo time (TE) = 2000/34 milliseconds, 128 × 128 matrix, and a ×30 30-mm field of view. Continuous 1-mm thick slices were used to cover the entire tumor region. Then, a T1 map was acquired with a gradient-echo multilip angle approach with the following parameters: TR/TE = 115/3 milliseconds, 128 × 128 matrix, a 30 × 30 mm field of view, number of excitations (NEX) = 4, and 7 flip angles of 10, 20, 30, 40, 50, 60, and 70 degrees. A total of five 1-mm thick slices were acquired to cover tumor ROI in an interleaved mode. DCE–MRI used the same acquisition parameters as those above but with the fixed flip angle of 30 degrees. Five baseline images were acquired before gadoxidoterol injection, and then 40 images were acquired after gadoxidoterol injection of 0.0267 mmol/mL over a period of 15 seconds with a total injection volume of 0.15 mL. A syringe pump (NE-1600, New Era Pump Systems, Inc.) was used to inject gadoxidoterol at a constant rate (0.01 mL/sec).

The reference region model was used to calculate volume transfer constant, \(K_{\text{trans}}\) (transfer rate from plasma to the extravascular–extracellular space), and rate constant, \(k_{\text{ep}}\) (rate parameter for transport from the extravascular–extracellular space to plasma), as described in our previous study (28). The tumor area was segmented from the anatomic MR images with the signal intensity difference between the ROI and background, whereas the intensity thresholds were determined manually. The \(K_{\text{trans}}\), \(k_{\text{ep}}\) and

\[
\text{Var/Size} = \frac{\sum_{i=1}^{N} \left( \frac{\text{Sig}_i - \text{<Sig>}}{\text{PDF}_i} \right)^2 \times \text{PDF}_i}{N},
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where Sig\(_i\) is the signal intensity on the pixel \(i\), \(<\text{Sig}>\) is the mean signal intensity of all pixels on the line, PDF\(_i\) is the probability density function on the pixel \(i\), and \(N\) is the total number of pixels on the line. PDF\(_i\) was calculated by

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where Sig\(_{\text{max}}\) is the maximum signal intensity on the line. Variance per size presents how randomly the fluorophore-labeled antibodies are distributed on the cell membrane and is smaller when the signal intensity is more focused on a restricted region. The signal intensities of fluorophores were measured by ImageJ (version 1.40g; NIH).
C_{40min} (gadoteridol concentration at 40 minutes after dosing) values were averaged in the whole tumor region. Tumor volume was calculated by summing all voxels inside the tumor boundary of the anatomical MR images. Segmentation of the whole tumor area was carried out by ImageJ (version 1.40g; NIH). The $k_{\text{trans}}$, $k_{\text{ep}}$, and $C_{40min}$ quantifications were implemented by computer software developed using Labview (version 8.5; National Instruments Co.).

**Biodistribution study**

Tumor and blood were collected from each animal of groups 3 and 4 at 24 hours after radioactivity injection. Those samples were weighed, and the Tc-99m activity was measured with a calibrated gamma-ray counter (MINAXiγ Auto-gamma 5000 series Gamma Counter manufactured by Packard Instrument Company), and corrected for radioactive decay to the time of injection. The administered activity for each mouse was determined by measurements of the dosing syringe before and after injection with an Atomlab 100 dose calibrator (Biodex Medical Systems). Tissue activity concentrations [in percent of the injected dose per gram (%ID/g)] and tumor to blood activity concentration ratios were derived.

**PET/CT Imaging**

PET/CT imaging study was conducted by Triumph, a PET/CT dual-modality imaging system (GE), to monitor tumor response following TRA-8 administration with/without anti-EMMPRIN therapy. For PET imaging, this system provided a 2.2-mm axial spatial resolution and 5.9% sensitivity at the center of field of view, whereas the axial field of view was 37.5 mm (30). Animals were fasted overnight (17–21 hours) before and after injection with an Atomlab 100 dose calibrator (Biodex Medical Systems). Tissue activity concentrations [in percent of the injected dose per gram (%ID/g)] and tumor to blood activity concentration ratios were derived.

PET/CT imaging study was performed by Triumph, a PET/CT dual-modality imaging system (GE), to monitor tumor response following TRA-8 administration with/without anti-EMMPRIN therapy. For PET imaging, this system provided a 2.2-mm axial spatial resolution and 5.9% sensitivity at the center of field of view, whereas the axial field of view was 37.5 mm (30). Animals were fasted overnight (17–21 hours) before $^{18}$F-FDG injection. $^{18}$F-FDG (7.19 ± 0.07 MBq in 200 μL PBS) was administered intravenously, and a 7-minute scan was done at 100 minutes after injection. Animals were under isoflurane anesthesia during dosing (~5 minutes) and imaging (~10 minutes); the animal cages were heated with a heating pad (TPump, Gaymar Industries, Inc.) set to 42°C and started at 1 hour before $^{18}$F-FDG injection and continued until the imaging of all animals was completed. The temperature of the animal bed in the Triumph scanner was maintained to 37°C during imaging. PET images were reconstructed with maximum likelihood expectation maximization algorithm (5 iterations) in high-resolution mode. For contrast-enhanced CT imaging, 0.2 mL of iohexol was intra-peritoneally injected right before PET imaging. The voltage of X-ray tube was 75 kVp, and the anode current was 0.11 mA. The axial field of view was set to 78.9 mm, while 256 projections were acquired in fly gantry-motion mode. The CT acquisition time was 1.07 minutes per each animal. The coli-registration of PET and CT images was performed by ImageJ (version 1.40g; NIH). The tumor area was manually segmented from the co-registered contrast-enhanced CT images based on the signal intensity difference between the ROI and background. In PET images, the standardized uptake value (SUV) was calculated by SUV = (C × W)/D where C is tissue activity concentration (MBq/mL), W is animal body weight (g), and D is the administered dose (MBq). Before dosing, the averaged tumor SUV$_{\text{mean}}$ of groups 5 to 8 (MIA PaCa-2 model) was 1.04 ± 0.07 g/mL, and the averaged tumor volume was 91 ± 8 mm$^3$, without statistical difference among groups, whereas the averaged tumor SUV$_{\text{mean}}$ of groups 9 to 12 (PANC-1 model) was 0.87 ± 0.04 g/mL, and the averaged tumor volume was 31 ± 2 mm$^3$, without statistical difference among groups.

**Histologic analyses**

Ki67 and CD31 staining were carried out for tumor tissues of groups 5 to 12, with the same procedure as reported previously (29, 31); no tumor cells were remained in one of the samples of the TRA-8–treated group (group 7), so only 5 samples were analyzed for group 7. Two digital microphotographs (×400) were randomly selected for each tumor slice that had undergone Ki67 or CD31 staining using SPOT camera on an Olympus 1 × 70 microscope (Olympus Optical Co.), interfaced with personal computer and SPOT software. The proliferating (Ki67-expressing) cells were segmented by the signal intensity difference between the target cells and background in each photograph, whereas the intensity and minimum particle size thresholds were determined manually. Then, the target cells were counted, and the cell density (cell number (N)/mm$^2$) was calculated. The cell densities of the 2 photographs for each tumor slice were averaged. The CD31-stained area was segmented in the same way, and the area fraction (CD31-stained area/total area), considered as endothelial cell density, was calculated. The image analysis was conducted by ImageJ (version 1.40g; NIH).

**Statistical analysis**

One-way ANOVA (32) was carried out using SAS (version 9.2; SAS Institute Inc.) to compare the fluorescence signal amplitudes, the variance of the fluorescence signal distributions, and %ID/g of tumor and blood together with tumor to blood ratio. ANOVA was also used to compare the changes of the $k_{\text{trans}}$, $k_{\text{ep}}$, and $C_{40min}$ and the densities of Ki67 and CD31 staining cells in tumors. SPSS (version 16.0; SPSS Inc.) was used to compare tumor SUV$_{\text{mean}}$ and volume changes over 2 weeks of groups 5 to 8 (or groups 9 to 12) using 2-way repeated measures analysis of variance (RM ANOVA; ref. 33), whereas the Pearson correlation coefficient was used to analyze the relationships between 2 variables (34). $P < 0.05$ was considered significant. Data are presented as means ± SE.

**Results**

TRA-8 showed high killing efficacy for both MIA PaCa-2 and PANC-1 cell lines in an in vitro ATPlile assay, while additional anti-EMMPRIN therapy did not improve the cytotoxicity

Figure 1 presents the in vitro viability of MIA PaCa-2 (Fig. 1A) and PANC-1(Fig. 1B) cells following overnight
treatment of TRA-8 with/without anti-EMMPRIN antibody in escalating concentration. TRA-8 monotherapy induced 91% and 64% killing of MIA PaCa-2 and PANC-1 cells, respectively, at 500 ng/mL, but the cytotoxicity was not improved by adding anti-EMMPRIN antibody. TRA-8 killing efficacy for MIA PaCa-2 was 87% ± 28% better than that for PANC-1, when averaged over the 3 TRA-8 concentrations (10, 50, and 500 ng/mL).

Cy5.5–TRA-8 generated cellular caps in both MIA PaCa-2 and PANC-1 cells, while Cy3–anti-EMMPRIN antibody was more uniformly distributed on the cell membrane.

Figure 2A and B shows representative fluorescence images of Cy5.5–TRA-8 (red) and Cy3–anti-EMMPRIN antibody (green) distributed on MIA PaCa-2 and PANC-1 cells, when the 2 antibodies were used separately (Fig. 2A) or combined (Fig. 2B). Figure 2C–F presents the signal intensities of Cy5.5–TRA-8 and Cy3–anti-EMMPRIN antibody on the yellow lines shown in the images of MIA PaCa-2 (Fig. 2C and E) or PANC-1 cells (Fig. 2D and F), when the 2 antibodies were used separately (Fig. 2C and D) or combined (Fig. 2E and F). The maximum and minimum signal intensities together with variance per size were summarized in Table 1. When added separately, the maximum signal intensity of Cy5.5–TRA-8 was 2.6-fold higher than that of Cy3–anti-EMMPRIN antibody in MIA PaCa-1 cells, whereas the variance per size was significantly smaller. Similar distribution patterns were observed when combined. However, of interest, Cy3–anti-EMMPRIN antibody was observed in clusters as found for Cy5.5–TRA-8 in MIA PaCa-2 cells; the capping region of Cy3–anti-EMMPRIN antibody corresponded to that of Cy5.5–TRA-8 (Fig. 2B). The maximum signal intensity of Cy3–anti-EMMPRIN antibody was significantly higher when combined with Cy5.5–TRA8 than when alone (P = 0.003), whereas the variance per size was significantly smaller in the combined treatment also (P < 0.001).

Anti-EMMPRIN therapy enhanced the tumor uptake of MR contrast agent, but not Tc-99m–TRA-8

Figure 3A shows the concentration of MR contrast (gadoteridol) of 2 representative mice bearing orthotopic pancreatic tumor xenografts at 0 (baseline), 5, and 40 minutes after gadoteridol injection, when the animals were untreated (control) or treated with anti-EMMPRIN antibody at 3 days before imaging. The tumor boundary is indicated with the white dotted circle. Figure 3B shows the contrast enhancement curves averaged in the ROI indicated with the white square (2 × 1 window: 2 pixels located in the tumor) in the baseline images of Fig. 3A, together with the best-fit fifth order polynomial curves. The MR contrast concentration in tumor of the control animal was markedly higher than that of the treated animal immediately after injection, but was rapidly reduced over time. On the contrary, the MR contrast concentration in the treated tumor was lower than control tumor immediately after injection, but gradually increased and was maintained higher than that of control tumor by 20 minutes postinjection. Figure 3C and D shows the tumor $K_{trans}$ and $k_{ep}$ maps of the 2 animals untreated or treated with anti-EMMPRIN antibody, respectively. The changes of intratumoral $K_{trans}$, $k_{ep}$ and $C_{40min}$ values were −36% ± 9%, −18% ± 12%, and 153% ± 44%, respectively, for 3 days after drug dosing, whereas those of the control group were 38% ± 8%, 10% ± 7%, and 14% ± 25%, respectively, during the same time (P < 0.001, 0.085, and 0.025, respectively). The tumor volume change of the treated group was 22% ± 6%, while that of the control group was 52% ± 21% without statistical difference (P = 0.316). In the biodistribution study, the %ID/g of Tc-99m–TRA-8 in tumor, blood, and tumor to blood ratio for the anti-EMMPRIN–treated group were 28% ± 5%, 17% ± 1%, and 1.8 ± 4 respectively, while those of the control group were 20% ± 5%, 19% ± 1%, and
1.1% ± 0.3% respectively. No statistically significant differences were observed between the 2 groups for any of these parameters.

**18**F-FDG PET/CT imaging validated the additive therapeutic efficacy of combined anti-EMMPRIN antibody and TRA-8 for pancreatic cancer

Figure 4A shows representative contrast-enhanced CT (CE-CT), 18F-FDG PET, and PET/CT fused images of a mouse bearing an orthotopic MIA PaCa-2 pancreatic tumor indicated with a white arrow in each subfigure. Figure 4B and C shows the changes of MIA PaCa-2 tumor volume and SUV<sub>mean</sub> for 2 weeks posttherapy initiation when groups 5 to 8 were untreated or treated with anti-EMMPRIN antibody, TRA-8, and the combination, respectively. The different Greek letters represent statistically significant differences among groups; that is, group labeled with “"a"” is significantly different from that with “"b"”, but not from that with “"a"”, “"b"”. At day 28, the combination therapeutic efficacy was fairly comparable with the sum of the effects of the 2 monotherapies relative to the control group in volume assessment (decrease of 187% vs. 185%), showing the additive effect of the combination. At the end of the imaging study, the mean tumor volumes of groups 5 to 8 were 308 ± 28, 274 ± 43, 16 ± 6, and 8 ± 4 mm<sup>3</sup>, respectively. The correlation between the changes of tumor volume and intratumoral SUV<sub>mean</sub> over 2 weeks of therapy was statistically significant (P < 0.001). The animal body weights of groups 5 to 8 were not different during 2 weeks of therapy.

Figure 4D and E shows the changes of PANC-1 tumor volume and SUV<sub>mean</sub> for 2 weeks posttherapy initiation when groups 9 to 12 were untreated or treated with anti-EMMPRIN antibody, TRA-8, and the combination, respectively. The different Greek letters represent statistically significant differences among groups. The combination therapy presented additive efficacy in volume assessment, but not as marked as that in MIA PaCa-2 model (decrease of 86% vs. 111% for a week). At the end of the imaging study, the mean tumor volumes of groups 9 to 12 were 79 ± 12, 47 ± 7, 45 ± 9, and 38 ± 7 mm<sup>3</sup>, respectively. The tumor volume and SUV<sub>mean</sub> changes over 2 weeks were significantly correlated (P = 0.004). The animal body weights of groups 9 to 12 were not different during 2 weeks of therapy.

Figure 2. In vitro fluorescence imaging of antibodies and image analysis. A and B, representative fluorescence images of Cy5.5–TRA-8 and Cy3–anti-EMMPRIN antibody with MIA PaCa-2 and PANC-1 cells when the 2 antibodies were used separately (A) or combined (B). C–F, the fluorescence signal intensities of Cy5.5–TRA-8 and Cy3–anti-EMMPRIN antibody along the yellow lines are shown in the images of MIA PaCa-2 (C and E) or PANC-1 (D and F) cells when the 2 antibodies were used separately (C and D) or combined (E and F).
Histologic analysis verified that anti-EMMPRIN antibody significantly suppressed the endothelial cell and proliferating cell densities in pancreatic tumors.

Figure 5A shows the representative microphotographs of Ki67-and CD31-stained tumor slices (5-μm thickness) of groups 5 to 8 (or groups 9–12) untreated or treated with anti-EMMPRIN antibody, TRA-8, and the combination, respectively for 2 weeks. The proliferating (Ki67 stained) and endothelial (CD31 stained) cells are indicated with black arrows in each subfigure. Figure 5B and C shows the averaged Ki67 and CD31 expressed tumor cell

<table>
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<th>Table 1. The maximum and minimum signal intensities and variance per size of Cy5.5–TRA-8 and Cy3–anti-EMMPRIN antibody distributed on MIA PaCa-2 or PANC-1 cells when the 2 antibodies were used individually or in combination</th>
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NOTE: P values for statistical comparison are given.
Abbreviations: Max, maximum signal intensity; Min, minimum signal intensity; TRA-8, Cy5.5-labeled TRA-8; Anti-EMMP, Cy3-labeled anti-EMMPRIN antibody.

Figure 3. In vivo DCE-MRI and image analysis. A, representative contrast concentration maps acquired from dynamic contrast-enhanced MRIs of 2 animals at 0 (baseline), 5, and 40 minutes after gadoteridol injection with the same intensity scale (from 0.4 to −0.1 mmol/L) when the 2 animals were untreated (control) or treated with anti-EMMPRIN antibody at 3 days before imaging. B, contrast-enhancement curves averaged in the ROI indicated with the white square (5 × 1 window: 2 pixels) shown in Fig. 3A, together with the best fitting fifth order polynomial curves. The boundaries of the 2 tumor regions are indicated with dotted circles in A, Ktrans (C) and Aexp (D) maps of the tumors of the 2 animals untreated (control) or treated with anti-EMMPRIN antibody at 3 days before imaging, whereas the dark central area is necrosis caused by vascular insufficiency.
densities of groups 5 to 12, respectively; the different Greek letters or alphabets above bars represent the statistical difference among groups 5 to 8 (MIA PaCa-2 model) or groups 9 to 12 (PANC-1 model), respectively. The mean tumor cell proliferations of groups 6 to 8 were reduced 34%, 37%, and 26% relative to the control (group 5), respectively, but the statistical significance was detected only between groups 5 and 6 ($P = 0.035$). Similarly, the mean intratumoral endothelial cell densities of groups 6 to 8 were reduced 46%, 37%, and 17% relative to the control, respectively, but only that of group 6 was significantly lower ($P = 0.009$). But, in PANC-1 tumors, the cell density proliferation was significantly suppressed by either anti-EMMPRIN therapy ($P = 0.044$) or combination therapy ($P = 0.016$), and the endothelial cell density was significantly reduced by any of the three treatments ($P < 0.05$).

Discussion

The super aggregation, or capping, of death receptors has been considered to play a pivotal role to induce cellular apoptosis; the importance of Fas capping for apoptosis induction was validated (35, 36), and it was also shown that DR5 aggregation triggered by TRAIL (tumor necrosis factor–related apoptosis-inducing ligand) was essential to drive intracellular signals for death (37, 38). Similarly, it was found that TRA-8 resistance resulted not from the total number of DR5 expressed on the cell membrane or TRA-8 binding affinity, but perhaps from the inefficient TRA-8/DR5 oligomerization (8). The compact binding of Cy5.5–TRA-8 will lead to the local increase of the fluorescence signal, whereas the lack of clustering may cause the signal dispersion. This is consistent with the data presented in this study; the
maximum signal intensity of Cy5.5–TRA-8 capping in MIA PaCa-2 cells was about 2- to 3-fold higher than that in PANC-1, whereas the variance per size was about 20% to 30% smaller, with statistical significance, which is fairly comparable with TRA-8 cytotoxicity to the 2 cell lines. Therefore, this technique may be applicable for biopsy specimens of pancreatic cancer during a neoadjuvant trial, to identify potential responders to TRA-8. The clustering of Cy3–anti-EMMPRIN antibody was also observed when used together with Cy5.5–TRA-8, although it did not form the capping when used alone. This is presumably because both EMMPRIN and DR5 may be present in the same lipid raft (39); EMMPRIN might be clustered along with DR5 aggregation, which is supported by the regional consistency in the distributions of Cy5.5–TRA-8 and Cy3–anti-EMMPRIN antibody when combined.

Anti-EMMPRIN therapy increased the tumor delivery of gadoteridol, presumably by reducing intratumoral microvasculature and thereby decreasing interstitial pressure. However, the uptake of Tc-99m–TRA-8 into tumor was not enhanced by anti-EMMPRIN therapy. This discrepancy may be caused by the difference in circulation half-lives of the 2 agents; the plasma half-life of TRA-8 is about 6 days (data not shown), whereas that of gadoteridol is only about 3 hours in healthy mice (24). Therefore, although high interstitial pressure is present in a tumor, TRA-8 may be eventually delivered into the target cells. In contrast, small molecules are rapidly excreted, unless they reach the target cells and then are internalized within the limited time period. These results may explain the additive efficacy in the combination therapy with anti-EMMPRIN antibody and TRA-8, and the synergistic efficacy when anti-EMMPRIN antibody used with gemcitabine.

Figure 5. Histologic analysis of tumor response. A, representative microphotographs of Ki67 and CD31 staining of MIA PaCa-2 or PANC-1 tumors after untreated (served as control) or treated with anti-EMMPRIN antibody, TRA-8, and combination for 2 weeks, respectively, whereas the proliferating and endothelial cells are indicated with black arrows in each subfigure. Proliferating (Ki67 positive; B) and endothelial (CD31 positive; D) cells densities in the 4 groups, whereas statistical differences among groups are indicated by different Greek letters (MIA PaCa-2) or alphabets (PANC-1) above bars.
(23). Of note, the hypervascular metastatic liver tumors from the primary pancreatic cancer are common (40); about 60% of pancreatic cancer patients have metastases to distant organs like liver and lung at diagnosis, and the complications due to local spread is one of the main causes of patient death. Therefore, enhancing drug delivery into hypervascular metastatic tumors while suppressing further tumor invasion is essential to significantly extend patient lives, and the anti-EMMPRIN antibody may be a good candidate to achieve that goal. Because no adverse side effect was observed during 2 weeks of anti-EMMPRIN monotherapy or combination therapy, anti-EMMPRIN antibody may be readily combined with other chemotherapeutic agents to maximize therapeutic efficacy.

Histologic analysis validated that anti-EMMPRIN antibody reduced the tumor cell proliferation and endothelial cell densities, but did not confirm the additive efficacy when combined with TRA-8 in MIA PaCa-2 tumor model. This may reflect that TRA-8 (or combination) therapy killed most cancer cells sensitive to the treatment, and thereafter, a small portion of cells inherently resistant (or acquired resistance) remained and could start dividing again. In fact, the averaged volume of PANC-1 tumors after 2 weeks of combination therapy was 5-fold larger than that of MIA PaCa-2 tumors after the same dosing, and the additive efficacy shown in PANC-1 tumor volume assessment was validated histologically. Therefore, to eradicate all tumor cells, the sequential use of several drugs having different therapeutic mechanisms would be ideal. 

18F-FDG PET/CT imaging enabled longitudinal evaluation of the combination therapy; because it allowed examining the entire tumor region noninvasively, it might be a better approach to assess tumor response than histologic analyses. However, because FDG is captured in proliferating cells as well as cancer cells, it would be hard to differentiate pancreatic cancer from pancreatitis based on 18F-FDG PET imaging. The higher specificity for targeting proliferating cells might be achieved by using 18F-labeled 3'-deoxy-3'-fluorothymidine (41), although its clinical usefulness will need to be validated using more investigations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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