Near-infrared photoimmunotherapy of pancreatic cancer using an indocyanine green-labeled anti-tissue factor antibody

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Abstract

**AIM**
To investigate near-infrared photoimmunotherapeutic effect mediated by an anti-tissue factor (TF) antibody conjugated to indocyanine green (ICG) in a pancreatic cancer model.

**METHODS**
Near-infrared photoimmunotherapy (NIR-PIT) is a highly selective tumor treatment that utilizes an antibody-photosensitizer conjugate administration, followed by NIR light exposure. Anti-TF antibody 1849-ICG conjugate was synthesized by labeling of rat IgG anti-TF monoclonal antibody 1849 (anti-TF 1849) to a NIR photosensitizer, ICG. The expression levels of TF in two human pancreatic cancer cell lines were examined by western blotting. Specific binding of the 1849-ICG to TF-expressing BxPC-3 cells was examined by fluorescence microscopy. NIR-PIT-induced cell death was determined by cell viability imaging assay. In vivo longitudinal fluorescence imaging was used to explore the accumulation of 1849-ICG conjugate in xenograft tumors. To examine the effect of NIR-PIT, tumor-bearing mice were separated into 5 groups: (1) 100 μg of 1849-ICG i.v. administration followed by NIR light exposure (50 J/cm²) on two consecutive days (Days 1 and 2); (2) NIR light exposure (50 J/cm²) only on two consecutive days (Days 1 and 2); (3) 100 μg of 1849-ICG i.v. administration; (4) 100 μg of unlabeled anti-TF 1849 i.v. administration; and (5) the untreated control. Semiweekly tumor volume measurements, accompanied with histological and immunohistochemical (IHC) analyses of tumors, were performed 3 d after the 2nd irradiation with NIR light to monitor the effect of treatments.

**RESULTS**
High TF expression in BxPC-3 cells was observed via western blot analysis, concordant with the observed preferential binding with intracellular localization of 1849-ICG via fluorescence microscopy. NIR-PIT-induced cell death was observed by performing cell viability imaging assay. In contrast to the other test groups, tumor growth was significantly inhibited by NIR-PIT with a statistically significant difference in relative tumor volumes for 27 d after the treatment start date [2.83 ± 0.38 (NIR-PIT)] vs 5.42 ± 1.61 (Untreated), vs 4.90 ± 0.87 (NIR), vs 4.28 ± 1.87 (1849-ICG), vs 4.35 ± 1.42 (anti-TF 1849), at Day 27, P < 0.05]. Tumors that received NIR-PIT showed evidence of necrotic cell death-associated features upon hematoxylin-eosin staining accompanied by a decrease in Ki-67-positive cells (a cell proliferation marker) by IHC examination.

**CONCLUSION**
The TF-targeted NIR-PIT with the 1849-ICG conjugate can potentially open a new platform for treatment of TF-expressing pancreatic cancer.

**Key words:** Pancreatic cancer; Anti-tissue factor antibody; Indocyanine green; Photoimmunotherapy; Near-infrared

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**Core tip:** We examined whether anti-tissue factor (TF) antibody 1849-indocyanine green (ICG) conjugate (1849-ICG) induced the photodynamic effect in a pancreatic cancer xenograft. There was no report about employing 1849-ICG conjugate which selectively binds the target antigen TF for near-infrared photoimmunotherapy (NIR-PIT) of tumor, though some studies have suggested the usefulness of anti-TF 1849 in cancer imaging and therapy. Our study proposes for the first time that 1849-ICG conjugate is a desirable candidate for new treatment modality NIR-PIT of pancreatic cancer after evaluating its cytotoxic and antitumor effects via in vitro and in vivo studies in mouse model of pancreatic cancer.

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INTRODUCTION
Pancreatic cancer is one of the most devastating health issues that has caused 411600 deaths, globally, in 2015 for all ages and both sexes[1]. In 2018, in the United States, it is the fourth and ninth leading cancer type for estimated cancer death and new cancer case, respectively[2]. Pancreatic cancer has the lowest 5-year survival rate of 8%, for all stages combined[3]. The major reasons of poor prognosis are late diagnosis and lack of effective therapy. Therefore, for achieving early diagnosis and new treatment options, the efficacious antibody based molecular-targeting therapeutic approaches are currently gaining attention in preclinical and clinical research. Conventional immunotherapy itself as well as using certain antibodies, antibody-drug conjugate (ADC) therapy, radioimmunotherapy (RIT), and photoimmunotherapy (PIT) are being investigated substantially. Meanwhile, the effort to explore a novel target molecule and a suitable theranostic agent is still imperative.

Tissue factor (TF) is a 47-kDa single chain transmembrane glycoprotein belonging to the cytokine receptor family group 2, composed of 263 amino acid residues. TF mediates a variety of physiologically- and pathophysiologically-relevant functions and its overexpression is linked to thrombogenicity, tumor angiogenesis, cell signaling, tumor cell proliferation, and metastasis[4-6]. Various malignant entities including pancreatic cancer has shown the expression of TF[6,7]. Moreover, in contrast
to normal pancreas with low TF expression, a high TF expression in pancreatic cancer correlates with tumor grade, extent, metastasis and invasion.\(^{[6,9]}\). Haas and co-workers have previously analyzed the expression of TF in eight human pancreatic cancer cell lines including BxPC-3 and reported presence of TF expression, at RNA and protein level. Corresponding to the TF expression in cell lines, they also demonstrated that most of the tissue specimens of pancreatic cancer patients have highly variable TF expression, as determined by immunofluorescence staining.\(^{[10]}\). Previously, we suggested that TF may be a promising target for cancer diagnostic imaging or therapy, developed several anti-TF antibodies, and showed that a rat IgG\(_a\) anti-TF monoclonal antibody 1849 has high affinity against TF.\(^{[11,12]}\) We reported the development of Alexa Fluor 647-labeled anti-TF antibody 1849 probe for fluorescence imaging in a TF-overexpressing human pancreatic cancer xenograft model\(^{[13]}\) and an 1\(^{11}\)In-labeled anti-TF antibody 1849 probe for immuno-spectroscopy imaging (SPECT) imaging in glioma model\(^{[13]}\) and pancreatic cancer models (manuscript under preparation). Cai et al.\(^{[14,15]}\) have successfully developed a radiotracer for immuno-PET (positron emission computed tomography) imaging of in vivo TF expression in pancreatic cancer and breast cancer models\(^{[16]}\). Wang et al.\(^{[17]}\) labeled anti-TF antibody with \(^{90}\)Y and reported its radiotherapeutic effect on human xenograft NSCLC tumors in nude mice. These studies that considered TF as a molecular target encourage us to use anti-TF antibody 1849 in near-infrared PIT (NIR-PIT).

NIR-PIT is a modified version of the conventional photodynamic therapy (PDT) or photothermal therapy (PTT). NIR-PIT exerts a target cell specific cancer treatment that enables highly selective cell death after systemic administration of a photosensitizer-conjugated antibody against tumor-associated antigens, and accompanying exposure with NIR light. The light of a specific wavelength activates the relevant photosensitizer, and this interaction induces a cytotoxic reaction. An antibody which binds a cancer specific antigen expressing on the cellular membrane, is desirable for selective targeting of cancer cells. Over recent decades, a wide variety of available monoclonal antibodies that bind to the various molecular targets, have been considered and used with NIR-PIT in preclinical studies by several research groups. Among them, NIR-PIT using anti-epidermal growth factor receptor (EGFR) antibody (cetuximab) conjugated to a photosensitizer, phthalocyanine dye IRDye\(_{700}\) NHS Ester (IR700DX), has been widely studied and is currently in its Phase I/II clinical study (study of RM-1929 and PIT in patients with recurrent head and neck cancer: NCT02429279).

In the present study, we conjugate an anti-TF monoclonal antibody 1849 with a photosensitizer, indocyanine green (ICG), and demonstrate the potential of the generated 1849-ICG conjugate as a desirable candidate for NIR-PIT of pancreatic cancer, after evaluating its cytotoxic and antitumor effects via in vitro and in vivo studies in a mouse model of pancreatic cancer.

### MATERIALS AND METHODS

#### Cell lines

The human pancreatic cancer cell lines, BxPC-3 and SUIT-2, were purchased from the ATCC (Manassas, VA, United States). BxPC-3 cells were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, United States) supplemented with 10% fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan), 100 U/mL penicillin-G sodium, and 100 μg/mL streptomycin sulfate (Invitrogen, Carlsbad, CA, United States) at 37 °C under a humidified atmosphere containing 5% CO\(_2\). SUIT-2 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

#### Western blot analysis

Western blotting was performed to analyze TF expression in cultured cells. Whole-cell lysates were prepared using radioimmunoprecipitation assay buffer (Wako Pure Chemical Industries, Osaka, Japan) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Total protein concentration was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). Protein samples (35 μg) were separated on a 4%-20% polyacrylamide gel (ATTO Corporation, Tokyo, Japan) and transferred on to Immobilon-P membrane (Millipore, Billerica, MA, United States). As primary antibodies, anti-TF 1849 (generated by us) and a commercially available goat anti-human actin (C-11) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States), were used. A horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody and anti-rat IgG antibody (GE Healthcare, Little Chalfont, United Kingdom) were used as the secondary antibodies. Immunoreactive bands were visualized using the Enhanced Chemiluminescence Plus Western blotting detection system (GE Healthcare).

#### Photosensitizer labeling of anti TF-antibody

The rat IgG\(_b\) anti-TF monoclonal antibody 1849 which reacts with human TF antigen was developed as previously described\(^{[12]}\). An ICG Labeling kit-NH\(_2\) was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD, United States). Labeling of antibody with ICG was performed according to the manufacturer's manual. A component of this kit, NH\(_2\)-reactive ICG, has a succinimidyl ester group and can easily form a covalent bond with an amino group of the antibody. Briefly, NH\(_2\)-reactive dyes were added to anti-TF antibody (130 μg) solution on the membrane of a filtration tube and incubated at 37 °C for 10 min. The buffer solution was then added to the mixture and centrifuged. The conjugate was recovered by pipetting with phosphate-buffered saline (PBS). The concentration of the conjugates was determined...
by Bradford protein assay using a BioRad microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The absorbance of ICG was measured at 800 nm with the Synergy HT multi-mode microplate reader (BioTek Instrument, Inc., Winooski, VT, United States), and the number of fluorophore molecules conjugated to each antibody was calculated. The ratio of ICG to antibody was 0.4.

**Fluorescence microscopy studies**

Fluorescence microscopy was employed to observe the localization of 1849-ICG and the cytotoxic effect of PIT. BxPC-3 or SUIT-2 cells were plated on a Lab-Tek Chamber slide (Thermo Fisher Scientific, Frederick, MD, United States) and incubated for 18 h. Subsequently, culture media were discarded, and the cells were immediately stained after fixation with cold methanol for 5 min at -20°C. Non-specific binding of the antibody was blocked by incubating with Block-Ace reagent (Dainippon Pharmaceutical, Osaka, Japan) solution containing 10% goat serum for 30 min at room temperature. Subsequently, the cells were incubated with the 1849-ICG diluted (1:100) in antibody diluent buffer (Dako, Glostrup, Denmark) or without antibody, at 4°C. After 18 h, cells were washed with Tris-buffered saline (TBS), and the nuclei were stained with 4,6-diamidino-2-phenylindole Fluoromount-G (SouthernBiotech, Birmingham, AL, United States). The cells were visualized under a Keyence BZ-X700 microscope (Keyence Japan Co, Ltd, Osaka, Japan) equipped with the following filter sets: excitation wavelength 675 to 750 nm, emission wavelength 760 to 860 nm and dichroic mirror wavelength 760 nm for ICG. To compare BxPC-3 and SUIT-2 cells, images were acquired using the same settings of exposure time and black balance. Phase-contrast images were also acquired.

**Cell viability imaging assay after PIT**

BxPC-3 cells (2 × 10^4/well) were seeded in a chamber slide. After 6 h, the culture medium was changed to fresh medium containing 1849-ICG (2.5 μg/mL medium) or not. After an overnight incubation, the culture medium was replaced with phenol red free medium and the cells were subsequently irradiated with NIR light from an Infrared Diode Laser system (Laser Create Co., Tokyo, Japan) at a wavelength of 808 ± 3 nm and a power density of 50 J/cm² (1 W/cm² for 50 s). The irradiation dose was measured with a thermal laser power sensor and an optical power meter, Starlite (Ophir Japan, Saitama, Japan). Two hours later, two color staining assay using ReadyProbes Cell Viability Imaging kit (Thermo Fisher Scientific) was performed. As per manufacturer’s instruction, nuclei of all viable cells were stained with NucBlue Live reagent, while only the nuclei of dead cells were stained with NucGreen Dead reagent. The cells were imaged under a Keyence BZ-X700 microscope (Keyence Japan Co, Ltd) equipped with appropriate filter sets for DAPI (excitation/emission maxima: 360/460 nm), GFP (green) filter set (excitation/emission maxima: 470/525 nm) and ICG (excitation/emission maxima: 710/810 nm).

**Animal and tumor model**

A single-cell suspension of 5 × 10^6 BxPC-3 cells in 100 μL RPMI medium was mixed with BD Matrigel matrix (BD Biosciences, Bedford, MA, United States) and subcutaneously inoculated into both thighs of 7-wk-old male, BALB/cA Jcl-nu/nu nude mice (CLEA, Shizuoka, Japan) for in vivo imaging and NIR-PIT studies. During the experimental procedure, mice were anesthetized with isoflurane. All animal experiments were conducted in compliance with the guidelines for animal experimentation approved by the Animal Care and Use Committee of our institution, National Institute of Radiological Sciences.

**In vivo NIR fluorescence imaging**

Tumor-bearing mice were injected intravenously with 1849-ICG (100 μg) via a tail vein. The mice were anesthetized by inhalation of 2.5% isoflurane, and spectral fluorescence images at dorsal position were obtained using the Maestro In-vivo Imaging system (CRi, Woburn, MA, United States) with the ICG filter sets (excitation, 700–770 nm and emission, 790 nm long pass) at pre-injection, followed by various time points post-injection (24, 48, 72, and 144 h). The tunable filter was automatically stepped up in 10-nm increments from 780 to 950 nm for the ICG filter setting, while the camera sequentially captured images at each wavelength interval. With the commercial Maestro software (CRI), subtractions of background and baseline intensities, followed by un-mixing of spectral fluorescence images were performed a step by step. By setting the auto calculate threshold, the region of interest on entire tumors of the ICG spectrum image were determined and the tumor fluorescence signal intensities (FI) were measured. The constant exposure time was set to compare the FI of the serial images. Overlaid image of ICG spectrum image and white light image was acquired by using Photoshop software (Adobe, San Jose, CA, United States).

**In vivo PIT of tumor**

When the subcutaneous BxPC-3 tumors in mice reached approximately 10 mm at the longest diameter, the tumor-bearing mice were randomly assigned to one of the 5 groups (n = 6 for each group) for the treatment study conducted as per the shown scheme (Figure 1). Mice were initially injected 1849-ICG (100 μg) intravenously. On day 1 and day 2, the tumor was irradiated with NIR light from an Infrared Diode Laser system (Laser Create Co.) at a wavelength of 808 ± 3 nm and a power density of 50 J/cm² (1 W/cm² for 50 s), as measured with a StarLite Laser Power meter (OPHIR Japan Ltd.). During irradiation, the surrounding areas of tumors were shielded from light using aluminum foil. Briefly, group (1) received 1849-ICG i.v. administration followed by two doses of irradiation on two consecutive days, group (2)
received two doses of irradiation only on two consecutive
days, group (3) received 1849-ICG i.v administration
only, group (4) received unlabeled anti-TF 1849 i.v. ad-
ministration, and group (5) received no treatment. To
determine the tumor response, tumors of mice (n = 12
for each group except n = 11 in anti-TF 1849 group)
were measured twice a week throughout the experiment
using calipers, and volumes were approximated using
the formula: volume (mm$^3$) = [length (mm)]$\times$ [width
(mm)]$^2$ $\times$ 0.5. Relative tumor volume was calculated as
the volume on the indicated day divided by the starting
volume on the day prior to the treatment. Body weight
of mice was also measured twice a week; general health
conditions and local skin condition at the irradiated area
of mice were monitored daily.

**Histological and immunohistochemical analysis**
At 72 h after 2nd irradiation of NIR-PIT, 2 mice from each
group (n = 2) were euthanized by cervical dislocation
under anesthesia (isoflurane). Tumors were excised from
these mice, fixed in 4% paraformaldehyde, and
embedded in paraffin. To observe the histologic changes,
paraffin-embedded tissues were cut into 5-μm slice,
serial sections were fixed on glass slides and stained with
hematoxylin and eosin (H&E) staining. To perform
immunohistochemical (IHC) analysis, tissue sections
were rehydrated, and subjected to antigen retrieval.
The sections were stained for Ki-67, a cell proliferation
marker, using an anti-human Ki-67 polyclonal antibody
(Dako Denmark, Glostrup, Denmark), as previously de-
scribed. Slides were analyzed using the Olympus BX43
microscope system (Olympus, Tokyo, Japan).

**Statistical analysis**
All results were expressed as mean ± SD. Significant
differences between groups were determined by Student’s
t-test (Excel, Microsoft, Redmond, WA, United States).
Two-tailed unpaired t-test was used for comparisons of
relative tumor volume. The probability of survival based
on relative tumor volume (3.1-fold increase of individual
tumor volume from the initial volume) was estimated in
each group with a Kaplan-Meier survival curve analysis,
and the results were compared with use of the Gehan-
Breslow-Wilcoxon test. P-values < 0.05 were considered
significant.

**RESULTS**

**Expression of TF in human pancreatic cancer cell lines**
Expression of human TF protein in BxPC-3 and SUIT-2
pancreatic cancer cells was determined through western
blot analysis and fluorescence microscopy. In western
blot analysis of cell lysate, TF expression was found to be much higher in BxPC-3 cells than in SUIT-2 cells (Figure 2A). Moreover, fluorescence microscopy after overnight incubation with 1849-ICG revealed that the preferential binding with intracellular localization of 1849-ICG was higher in BxPC-3 cells than SUIT-2 cells (Figure 2B). Taken together, our data indicated the specific binding of the 1849-ICG conjugate antibody to the highly-expressed TF in BxPC-3 pancreatic cancer cells.

**Phototoxic cell death in response to TF-ICG mediated PIT**

PIT induced rapid cell death, and numerous dead cells stained with NucGreen® Dead reagent were visualized (Figure 3). Meanwhile, there was no significant cytotoxicity associated with exposure of 1849-ICG or NIR light exposure alone, in the absence of each other (Figure 3).

**In vivo tumor visualization by NIR fluorescence imaging**

Mice with subcutaneous BxPC-3 tumors were intravenously injected with 1849-ICG, followed by NIR fluorescence imaging at the indicated time points (24, 48, 72, 96 and 144 h after injection). In the untreated group, tumors showed highest FI 24 h after injection and fairly retained the FI until 144 h, our last observation time.
point, even though FI of tumor decreased gradually over time in longitudinal imaging (Figure 4A). In contrast, the FI of tumors in the PIT group decreased immediately after receiving the irradiation but it increased again in 24 h (Figure 4B).

**PIT effects on the tumor growth in nude mice**

Tumor growth was significantly inhibited by NIR-PIT with statistically significant differences in the relative tumor volumes for 27 d after the treatment onset when compared to the other groups (no treatment, 1849-ICG only, anti-TF 1849 only and NIR irradiation only) ($P < 0.05$) (Figure 5A). No significant therapeutic effect was observed in the control groups. A significantly prolonged survival was observed in the NIR-PIT group ($P < 0.05$ vs the other groups) (Figure 5B). No body weight change (Figure 5C), skin damage, or abnormal general condition were observed in these mice.

**Evaluation of the PIT effects with histological and IHC analysis**

According to our histological examination and some previous studies, the cells in BxPC-3 tumors have been observed to form a nest pattern, surrounded by stromal tissues in BxPC-3 tumors\textsuperscript{[20]}. However, H&E-stained sections of tumors treated with PIT revealed necrotic death-associated features, such as loss of tumor cells and the nest pattern, scattering of damaged and hypertrophic tumor cells, and marked interstitial fibrosis (Figure 6). On the other hand, no obvious damage was observed in the tumors of the control groups: receiving only 1849-ICG but no NIR irradiation, receiving only NIR irradiation, receiving only unconjugated anti-TF 1849, and receiving no treatment (Figure 6). As per the IHC examination, the numbers of Ki-67-positive tumor cells, indicating active proliferation, decreased noticeably in the tumor tissues of mice treated with PIT, when compared with the other groups (Figure 7).

**DISCUSSION**

For cancer therapy, conventional PDT has been suggested as one of the treatment modalities\textsuperscript{[21]}. In PDT, tumor targeting of photosensitizers depends primarily upon the passive enhanced permeability and retention (EPR) effect\textsuperscript{[22]}. However, EPR-based tumor targeting alone faces intrinsic limitations in its specificity and efficacy\textsuperscript{[23]}. To overcome this ambiguity, active targeting strategies using special molecules (antibodies, ligands, etc.), which bind to tumor antigens specifically, have been exploited and are preferred as a drug delivery system. Recently, Mitsunaga et al\textsuperscript{[24]} and others recommended cancer cell-selective NIR-PIT to overcome the limitation of conventional PDT. Their studies revealed that NIR-PIT induces reliable cytotoxic and antitumor effects in several tumors and derived cell lines\textsuperscript{[25-32]}. Researchers labeled the different types of tumor-specific antibodies such as anti-EGFR mAb\textsuperscript{[25,26]}, anti-CD20 mAb\textsuperscript{[27]}, anti-
CD44 mAb, anti-PD-L1 mAb, anti-PSMA mAb, anti-CD25 mAb, and anti-CEA mAb, with photosensitizer IR700DX, and used them as PIT agents. Although PIT is a promising therapeutic option, very few
PIT agents have been developed and examined. Therefore, there is a need to develop more PIT agents for treating various cancer patients.

High TF expression is commonly observed in a variety of solid tumors including pancreatic cancer and is associated with poor prognosis of pancreatic ductal adenocarcinoma[36]. Previously, we developed the anti-TF antibody 1849 (anti-TF 1849) alone (blue), for 27 d after the treatment start date. Values shown represent mean ± SD, aP < 0.05, NIR-PIT vs other groups (n = 12 for each group except n = 11 in anti-TF 1849 group); B: Significantly prolonged survival was observed in NIR-PIT group vs other groups, bP < 0.05, by Gehan-Breslow-Wilcoxon test (n = 12 for each group except n = 11 in anti-TF 1849 group); C: The average body weight did not differ significantly among all 5 groups of mice. Red arrowhead indicates the day of administration of 1849-ICG or Anti-TF 1849 and purple arrowheads indicate the day of NIR exposure. Anti-TF 1849: Anti-tissue factor antibody 1849; 1849-ICG: Indocyanine green-labeled anti-tissue factor antibody 1849; NIR: Near-infrared; PIT: Photoimmunotherapy.

Our western blot analysis demonstrated the higher expression of TF in BxPC-3 pancreatic cancer cells compared to the SUT-2 cells (Figure 2A). Other researchers have also reported the expression of TF in certain human pancreatic cancer cell lines[40]. These results encouraged us to use BxPC-3 as a representative TF-expressing pancreatic cancer model. Moreover, our results from in vitro and in vivo fluorescence imaging after overnight incubation of cells with 1849-ICG (Figure 2B) and tumor-bearing mice after 1849-ICG injection, respectively (Figure 4A), showed a strong ICG fluorescence signal in BxPC-3 cells and tumors; indicating the significantly increased accumulation of 1849-ICG in these cells and tumors. Therefore, we hypothesized that our pancreatic cancer xenograft expressed elevated levels of TF and would be bound specifically with 1849-ICG. Accumulation of 1849-ICG in tumors with fluorescence...
Aung W et al. PIT of pancreatic cancer with TF-ICG

Figure 6 Histological examination of effects of near-infrared photoimmunotherapy 72 h after 2nd exposure to near-infrared light. Tumors were resected and stained with hematoxylin and eosin. After near-infrared photodynamic therapy (NIR-PIT), tumors showed necrotic death-associated features including loss of tumor cells and cells nest pattern, scattering of damaged and hypertrophic tumor cells, and abundant fibrosis. No obvious damage was detected in the tumors of other control groups: receiving no treatment, NIR light alone, indocyanine green-labeled anti-tissue factor (TF) antibody 1849 (1849-ICG) alone and anti-TF antibody 1849 (anti-TF 1849) alone. Photos of tumor section were taken under 20 × objective magnification (scale bar = 100 μm). Anti-TF 1849: Anti-tissue factor antibody 1849; CTR: Control; 1849-ICG: Indocyanine green-labeled anti-tissue factor antibody 1849; NIR: Near-infrared; PIT: Photodynamic therapy.

Figure 7 Ki-67 immunostaining of tumor sections 72 h after 2nd exposure to near-infrared light in near-infrared photoimmunotherapy. A marked reduction in Ki-67-positive cell numbers was observed in tumors treated with near-infrared photodynamic therapy, compared with those of other control groups. Tumor section photos were taken under 20 × objective magnification (scale bar = 100 μm). Anti-TF 1849: Anti-tissue factor antibody 1849; CTR: Control; 1849-ICG: Indocyanine green-labeled anti-tissue factor antibody 1849; NIR: Near-infrared; PIT: Photodynamic therapy.

Image was serially quantified and was found to peak 24 h after injection. Following NIR-PIT, the ICG signal intensity in the tumor decreased initially, probably due to partial photobleaching of probe and cancer cell death.
Subsequently, the signal intensity tended to increase again, 24 h after PIT, indicating re-accumulation of circulating 1849-ICG in the surviving cells of the residual tumor. This phenomenon may ensure the effect of PIT by additional exposure of NIR 24 h later. Our present study is the first ever report of NIR-PIT targeting TF with 1849-ICG, although there have been studies regarding the targeting of TF for diagnostic imaging\cite{14-16} or radioisotope therapy\cite{17}. Since TF is expressed not only on tumor cell surface, but also, in tumor stroma\cite{37} and on tumor-associated vascular endothelial cells\cite{38}, our NIR-PIT with 1849-ICG may possibly target all of them.

As the NIR-PIT regimen, we used a single 1849-ICG injection followed by NIF light exposure on two consecutive days. We intravenously injected the 100 μg 1849-ICG conjugate in 100 μL PBS to mice and irradiated the tumor with NIR light (50 J/cm²) on two consecutive days. Even with this treatment regimen, tumor growth retardation was seen, and we could potentially obtain more optimal therapeutic efficacy with minimal adverse effects by increasing the injected dose of conjugate, the irradiation energy, fractioned doses and repeated treatment cycles. There have been reports that NIR-PIT, quickly and massively, kills target-expressing cancer cells within minutes after the NIF light exposure by provoking irreversible cellular membrane damage. As a result, extracellular water can enter the cells, resulting in swelling, blebbing, and rupture of the cells\cite{24}. Moreover, antibody-photosensitizer conjugates selectively bind to target-positive cancer cells with insignificant non-target cellular uptake of the photosensitizer, inducing rapid necrotic damage, especially on cancer cells near blood vessels. Thus, it causes the dilatation of tumor blood vessels and dramatically increasing permeability so called the super EPR (SUPR) effect\cite{25-41}. By means of this phenomenon after the initial NIR-PIT, antibody-photosensitizer conjugates can infiltrate more into the tumor microenvironment with enhanced permeability and penetration after the first dose of NIR-PIT, with a more homogenous access to the surviving cancer cells in tumors\cite{42}. Therefore, an additional irradiation with NIR light can further augment the therapeutic efficacy of NIR-PIT\cite{43}.

To comprehensively evaluate the potential effects of 1849-ICG directed NIR-PIT, we performed in vitro cell viability imaging assay, longitudinal monitoring of tumor volume change and histological and IHC analyses of tumor sections. NIR-PIT-induced rapid cell death was seen by fluorescence microscopy (Figure 3). Relative tumor volume change showed significant inhibition of tumor growth in the NIF-PIT group compared to the control groups (Figure 4A). In the histological investigation, H&E-stained NIR-PIT-treated tumor sections exhibited necrotic death-associated features (were loss of tumor cells and cells nest pattern, scattering of damaged and hypertrophic tumor cells, and marked interstitial fibrosis), whereas no obvious damage was observed in tumors of the other groups (Figure 6). Furthermore, in the IHC examination, a decrease in number of proliferating cells was noticed in the tumors of mice treated with NIF-PIT (Figure 7). Although rapid and massive cell death was induced by NIR-PIT, to obtain the optimal and prolong antitumor effect, further studies with fractionated dosing of conjugate and repeated exposure of suitable irradiation doses and/or the strategic studies for combining it with other drugs or treatment modalities will be needed.

Kobayashi et al\cite{39} and others utilized the IR700DX as the photosensitizer that has photostability, water solubility, and salt tolerance. In the present study, we employed a water-soluble anionic dye ICG as the photosensitizer, which is an already approved fluorescence imaging agent for clinical use by the United States Food and Drug Administration, since a couple of decades because of its safety\cite{49}. Furthermore, the superior features of NIR properties of ICG over other visible fluorophores, such as less auto-fluorescence, better tissue penetration, and large stokes shifts that allow better rejection of excitation light and thus lower background fluorescence\cite{45}, encouraged us to use it for in vivo NIR imaging and NIT-PIT. NH₄-reactive ICG, that we employed here, can easily form a covalent bond with an amino group of anti-TF 1849 without any activation process. Previously, ICG has been reported to be an effective NIR light absorber for laser-mediated photodynamic/PTT\cite{46-48}. Reindl et al\cite{49} reported that ICG absorbs 800-nm NIR light, and nearly 90% of the absorbed light is converted to heat. Moreover, there were additional reports about that ICG induces photo-oxidative cell death mediated by a singlet oxygen\cite{50} and heat generation\cite{51}. Thus, photothermal effect and oxidative stress are involved in the mechanisms resulting in the anti-tumor effect of ICG-NIR therapy. The present study demonstrated that ICG is useful for PIT, suggesting that ICG-labeled antibodies are alternative option in addition to IR700DX-labeled.
is a possibility to overcome it with advancing software and hardware technologies.

In conclusion, the current study reveals that 1849-ICG conjugate accumulated in a TF-overexpressing pancreatic cancer cell line and xenografts model BxPC-3. A single dose of 1849-ICG conjugate administration accompanied by NIR light exposure (50 J/cm²) for two consecutive days effectively inhibited tumor growth in vivo without producing any noticeable adverse effects. Taken together, our results suggest that NIR-PIT employing TF as a targeting antigen for the 1849-ICG is a promising new treatment modality for pancreatic cancer with a useful contemporaneous diagnostic utility, which could be ultimately applicable in clinical use.

ARTICLE HIGHLIGHTS

Research background
Pancreatic cancer is still one of major life-threatening diseases. Therefore, there is an urgent need to explore early diagnostic and new therapeutic options. Near infrared photomediation (NIR-PIT) is a highly selective tumor treatment that utilizes antibody-photosensitizer conjugate systemic administration, accompanied by subsequent NIR light exposure. Tissue factor (TF) is a transmembrane protein and its overexpression is associated with increased tumor growth, tumor angiogenesis and metastatic potential in many malignancies, including pancreatic cancer.

Research motivation
Previously, we suggested that TF may be a promising target for cancer diagnostic imaging probe and have already reported the usefulness of anti-TF monoclonal antibody (anti-TF mAb) in cancer imaging and therapy. However, NIR-PIT using anti-TF mAb has not been attempted.

Research objectives
In this study, we aim to investigate the photoimmunotherapeutic effect induced by a rat IgG anti-TF monoclonal antibody 1849 (anti-TF 1849), conjugated to the NIR photosensitizer, indocyanine green (ICG), in a TF-expressing BxPC-3 pancreatic cancer model.

Research methods
An ICG-labeled antibody conjugate (1849-ICG) was generated by labeling an anti-TF 1849 with ICG. The expression levels of TF in two human pancreatic malignancies, including pancreatic cancer.

Research results
High TF expression in BxPC-3 cells was observed via western blot analysis, concordant with the observed preferential binding with intracellular localization of 1849-ICG via fluorescence microscopy. NIR-PIT-induced cell death was observed by performing cell viability imaging assay.

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