Research Article

Near-infrared Photoimmunotherapy Targeting Cancer-Associated Fibroblasts in Patient-Derived Xenografts using a Humanized anti-Fibroblast Activation Protein Antibody

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Running title: NIR-PIT using Sibrotuzumab in PDX models

Keywords: cancer associated fibroblasts (CAFs); fibroblast activating protein (FAP); photoimmunotherapy (PIT); patient derived xenograft (PDX); Sibrotuzumab

Additional information:

Financial support: This work was supported by JSPS KAKENHI Grant Numbers JP20K09009 and JP21K08754.

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Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.

Word count:

Tables and figures: 6 figures (main article), 7 figures, and 2 tables (supplementary information)

Abstract

Esophageal cancer remains a highly aggressive malignancy with a poor prognosis, despite ongoing advancements in treatments such as immunotherapy. The tumor microenvironment, particularly cancer-associated fibroblasts (CAFs), plays a crucial role in driving the aggressiveness of esophageal cancer. In a previous study utilizing human-derived xenograft models, we successfully developed a novel cancer treatment that targeted CAFs with near-infrared photoimmunotherapy (NIR-PIT), as an adjuvant therapy. In this study, we sought to translate our findings toward clinical practice by employing patient-derived xenograft (PDX) models and utilizing humanized monoclonal antibodies, specifically Sibrotuzumab, which is anti-human fibroblast activation protein (FAP) antibody and already being investigated in clinical trials as monotherapy. PDX models derived from esophageal cancer patients were effectively established, preserving the expression of key biomarkers such as EGFR and FAP, as observed in primary tumors. The application of FAP-targeted NIR-PIT using Sibrotuzumab, conjugated with the photosensitizer IR700DX, exhibited precise binding and selective elimination of FAP-expressing fibroblasts in vitro. Notably, in our in vivo investigations using both cell line-derived xenograft and PDX models, FAP-targeted NIR-PIT led to significant inhibition of tumor progression compared to control groups,

all without inducing adverse events such as weight loss. Immunohistological assessments revealed a substantial reduction in CAFs exclusively within the tumor microenvironment of both models, further supporting the efficacy of our approach. Thus, our study demonstrates the potential of CAF-targeted NIR-PIT employing Sibrotuzumab as a promising therapeutic avenue for clinical treatment of esophageal cancer patients.

Introduction

Esophageal cancer is the eighth most prevalent cancer and the sixth most common cause of cancer death worldwide, with an estimated 604,000 new cases and 544,000 deaths from esophageal cancer in 2020 (1). The standard treatment for esophageal cancer is a combination of surgery, chemotherapy, and radiation therapy, which has recently expanded to include immunotherapy. Despite continuing developments and improvements in treatment for esophageal cancer, the outcome remains generally poor, with overall 5-year survival rates of less than 10%, and 5-year survival rates after esophagectomy of 15-40% (2). New therapeutic approaches are needed for this disease.

The tumor microenvironment (TME) plays an essential role in influencing tumorigenesis and tumor proliferation (3). The TME is composed of many types of cells, including endothelial cells, tumor-infiltrating lymphocytes, myeloid-derived suppressor cells, and tumor-associated macrophages. Among these cells, cancer-associated fibroblasts (CAFs) are often the most abundant (4). Our previous studies have demonstrated that CAFs in esophageal cancer tissue impact tumor proliferation, angiogenesis, immunosuppression, migration, invasion, metastasis, and treatment resistance (5-10). CAFs can stimulate cancer cells and remodel the TME by secreting

signaling molecules, including various inflammatory cytokines and growth factors, that support tumorigenesis and tumor progression (5, 11, 12). Fibroblast activation protein (FAP), a type II integral membrane serine protease of the prolyl oligopeptidase family, is known as a specific marker of CAFs, but is minimally expressed in normal fibroblasts (13, 14). Such properties make FAP a valuable targeting antigen for killing CAFs. Systemic cancer therapies targeting CAFs against FAP-positive cells have already reported some therapeutic benefits (15-18). However, because of their systemic nature, serious systemic side effects, such as cachexia and anemia, have been observed due to the expression of FAP in human bone marrow-derived mesenchymal stem cells (19). A safer yet effective, CAF-targeted cancer therapy would be highly desirable.

Near infrared photoimmunotherapy (NIR-PIT) is a novel cancer therapy that is based on the conjugation of a monoclonal antibody (mAb) to a photoabsorber (IR700 dye) followed by the application of ~690 nm NIR light irradiation (20). The antibody-photoabsorber conjugates (APC) are photochemically converted from highly hydrophilic to hydrophobic immediately after the irradiation of NIR light, leading to lipid-protein aggregation that places physical stress on the cellular membrane and impairs membrane function (21, 22). After light application, membrane damage allows the water outside of the cell to flow in, resulting in swelling and then bursting of the cell

(23, 24). Cellular contents are released into the TME which is highly immunostimulatory. Clinical trials in Phase 1/2a employing near-infrared photoimmunotherapy (NIR-PIT) targeting the epidermal growth factor receptor (EGFR) in patients with inoperable head and neck cancer have revealed clinically meaningful efficacy while maintaining a tolerable side effect profile. Encouraged by the positive outcomes of these clinical trials, the first EGFR-targeted NIR-PIT drug (Akalux®, Rakuten Medical Inc., San Diego, CA, USA), in conjunction with a 690 nm laser system (BioBlade®, Rakuten Medical Inc.), received conditional approval from the Japanese Ministry of Health, Labor and Welfare in September 2020, enabling its utilization for general clinical purposes. Furthermore, a global Phase 3 clinical trial, designated for expedited review by the FDA, is currently underway (NCT03769506).(25).

In theory, NIR-PIT is optimally suited for addressing superficial tumors, as NIR light typically penetrates tissue to a depth of only about 1–2 cm from the tissue surface. To address this limitation, a solution is offered by the utilization of fiber optic light diffusers that can be introduced within needle catheters for the treatment of deep-seated tumors, a technique that has already demonstrated its clinical feasibility. Moreover, NIR light can be delivered via endoscopic means, with the efficacy of endoscopic NIR-PIT having been validated in animal studies. Consequently, NIR-PIT holds the potential for

application in the treatment of both superficial and deep-sited tumors. By changing the antibody in the APC, NIR-PIT can be used in a variety of cancers each expressing different antigens either on the cancer cell or in the TME (26-29). Therefore, we developed a CAF-targeted NIR-PIT using an anti-human FAP antibody in a xenograft model, resulting in successful tumor regression (8, 10, 24).

Patient-derived xenografts (PDXs) are highly valuable tools for understanding tumor biology, evaluating therapeutic efficacy, and predicting drug responses, ultimately contributing to the development of precision approaches in cancer treatment (30-32). PDX models have the advantage over cell line-derived xenografts (CDXs) of including the actual TME. Since PDX models can preserve the TME of the original tumor, including such cells as fibroblasts, immune cells, blood vessels, and extracellular matrix components they are not only potentially more realistic but also offer more targets than CDXs. These features make PDX model valuable tools for translational research and precision medical approaches in cancer.

To apply CAF-targeted NIR-PIT in a clinical setting, we used Sibrotuzumab (33), a humanized mAb that binds FAP which has passed a phase 1 clinical trial for safety (34, 35). In this study, we reveal the therapeutic effect of CAF-targeted NIR-PIT using Sibrotuzumab in both CDX and PDX models of esophageal cancer.

Materials and Methods

Study approval

This study was conducted in accordance with the Declaration of Helsinki's ethical standards and the ethical guidelines for medical and health research involving human subjects. All cases were de-identified and details were removed from the case descriptions to ensure anonymity. The outline of our study was published on our webpage to explain the study and provide opportunities for disagreement. Owing to its retrospective nature, we requested and received permission to waive informed consent from the ethics committee. The use of clinical samples and procedures to isolate esophageal tissue-associated fibroblasts was approved and reviewed by the Ethics Review Board of Okayama University (No. 1707-022 and 1801-023; Okayama, Japan). Mouse experiments were conducted in a specific pathogen-free environment at the Okayama University Animal Facility in accordance with the institutional guidelines, and all animal experimental protocols were approved and reviewed by the Ethics Review Committee for Animal Experiments at Okayama University (OKU-2018327, OKU-2018790, OKU-2021191). All experiments were conducted in accordance with the guidelines and regulations of the committee.

Patients and tissue sample

A total of 149 esophageal cancer patients who underwent radical esophagectomy at Okayama University Hospital between 2008 and 2010 were enrolled. Patients were excluded if they: i) underwent follow up procedures; ii) were diagnosed with distant metastases or melanoma; or iii) were in remission after neoadjuvant therapy. In total, 140 patients were reviewed for age, gender, tumor location, histological type, the status of neoadjuvant therapy, tumor depth (pT), and lymph node status (pN). Tumor classification was applied according to the TNM Classification of Malignant Tumors 8th edition (UICC 8th edition). The use of clinical samples was approved and reviewed by the ethics review board of Okayama University, Okayama, Japan (No. 1801–023).

Immunohistochemistry for FAP in clinical specimens

Formalin-fixed paraffin-embedded (FFPE) surgical specimens of esophageal cancer sections of 3 μm thickness were dewaxed and soaked in 0.3% H_2O_2 in methanol at room temperature (RT) for 10 minutes to quench endogenous peroxidase activity. Antigen retrieval was performed by heating specimens in Tris/EDTA buffer pH 9.0 using a microwave. The sections were incubated with anti-FAP antibody (ab207178, clone

EPR20021, 1:500, Abcam, Cambridge, UK) for 120 minutes at RT. Following washes with PBS, sections were incubated with Envision+ anti-rabbit antibody (Dako) for 30 minutes at RT. The chromogen was liquid DAB+ (Dako). Sections were counterstained with Meyer's hematoxylin.

Evaluation of FAP area index

Tumor location was confirmed using hematoxylin and eosin (H-E) staining under microscopy at 12.5× magnification. FAP scoring was evaluated using an "Area Index", calculated by ImageJ software (http://rsb.info.nih.gov/ij/). To ensure that the whole tumor was evenly evaluated, at least two or more fields of tumor area were carefully selected at 40× magnification to evaluate FAP-positive CAFs. The mean value obtained from each sectioned tissue was defined as the FAP area index. All evaluations were performed by an independent pathologist who was blinded to clinical information.

Synthesis of IR700-conjugated antibodies

The conjugation of dyes with mAbs has been reported previously. Sibrotuzumab (1mg, Creative Biolab, TAB-211) was incubated with IRDye700DX N-hydroxysuccinimide ester (IR700; 63.5 μg, 5 mmol/L in DMSO, LICOR Biosciences, NE, USA) in 0.1

mol/L Na_2HPO_4 (pH 8.5) at 4℃ for 2 hours. The mixture was purified with a Sephadex G25 column (PD-10; GE Healthcare, IL, USA). We abbreviate Sibrotuzumab conjugated IR700 as Sib-IR700. In the same manner, Panitumumab (1 mg, Takeda Pharmaceutical Company, Tokyo, Japan) was incubated with IR700, and we abbreviate purified mixture as Pan-IR700. The protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad, CA, USA) by measuring the absorption at 595 nm using spectroscopy (SpectraMax i3, Molecular Devices). The concentration of IR700 was measured by absorption at 689 nm with spectroscopy (Nano Drop; Thermo Fisher, MA, USA) and the number of fluorophore molecules per Sibrotuzumab or Panitumumab was adjusted to approximately 3 (Supplementary Fig. S1). We performed SDS-PAGE as quality control for each conjugate as previously reported (36). We used diluted Sibrotuzumab and Panitumumab as non-conjugated controls for SDS-PAGE, and the fluorescent bands were measured with an IVIS Lumina (Xenogen, CA, USA) with a Cy5.5 filter set.

Cell lines

We used a human esophageal squamous cancer cell line (TE8), a human fetal esophageal fibroblast cell line (FEF3), and a human fetal lung fibroblast cell line (WI38). TE8 cells were purchased from the RIKEN BRC Cell Bank (Tsukuba, Japan)

(Nov 2018). WI38 cells were purchased from the Health Science Research Resource Bank (Osaka, Japan) (Oct 2011) and authenticated by JCRB Cell Bank (June 2019). FEF3 cells were isolated from the human fetal esophagus as described previously (5) and authenticated by JCRB Cell Bank (Jan 2018). Cells were frozen down soon after purchase or the authentication, and thawed to be used in this study. After thawing, cells were maintained in culture for no more than 30 passages.

Activation of fibroblasts by conditioned medium (CM) from cancer cell

TE8 cells were cultured in RPMI containing 10% FBS. After overnight attachment and growth in a flask, the cells were washed twice with PBS and then incubated in DMEM containing 2% FBS. The media was collected after 48-72 hours and centrifuged, defining it as CM. The CM is collected, frozen, and stored at − 30 °C until use. The CM was thawed for each experiment. FEF3 and WI38 cells were cultured with CM for 72-96 hours; the activated fibroblasts with CM were defined as CAF-FEF3 and CAF-WI38, respectively.

Western blotting

FEF3, CAF-FEF3, WI38, CAF-WI38, and TE8 were examined for the expression of

CAF markers by western blotting. The cells were homogenized and whole proteins extracted by centrifugation for 10 minutes at 4 ℃. The supernatant was collected and samples containing 30 mg of protein from each lysate was fractionated by 10% SDS-PAGE. Proteins were transferred to membranes and probed overnight at 4 ℃ with primary antibody against αSMA, FAP or β-actin (A5441, 1:1000, Sigma-Aldrich, MO, USA). TE8 were also examined for the expression of vimentin and pankeratin (1:1000). TE8 was examined for the expression of EFGR (1:1000). The membranes were washed in buffer and incubated with secondary antibodies for 1 hour at RT. After washing, the membranes were visualized using an Amersham Imager 600 (GE Healthcare).

Flow cytometry

To analyze the expression of FAP in the above cells, flow cytometry (FACS Lyric, BD Biosciences, NJ, USA) was used. The cells were labeled with PE-conjugated mouse anti-FAP antibody (R&D, FAB3715, 1:50, MN, USA) or Sib-IR700 (2.0 μg/mL) for 1 hour at 4 ℃ in the dark. PE-conjugated Mouse IgG1 isotype control (IC200P, R&D) was used as a control for anti-FAP antibody. Additionally, to confirm target specificity, an excess of unconjugated Sibrotuzumab (100 μg/mL) was added to the cells to block FAP before incubation with Sib-IR700.

Immunofluorescence microscopy

Fluorescence microscopy was performed using an IX83 fluorescence microscope (Olympus, Tokyo, Japan) to confirm the molecular target-specific localization of Sib-IR700 in FEF3, CAF-FEF3, WI38, CAF-WI38, and TE8. Cells were seeded on a 96-well black plate at 1.0×10^3/well. These cells were incubated with 20 μg/mL of Sib-IR700 for 6 hours at 37 ℃. After incubation, the cells were washed with PBS, and FluoroBright DMEM (Thermo Fisher) with propidium iodide (PI; 1:2000, Sigma-Aldrich) was added to identify dead cells. The cells were irradiated with NIR light at 25 J/cm^2 (L700-05AU 700 nm, Epitex Co, Kyoto, Japan) and the morphological and fluorescent changes were observed before and after treatment with the WIG filter setting for PI and the Cy5 filter setting for IR700. The power density of 30 mW/cm^2 was measured with an optical power meter (PM 100; Thorlabs, NJ, USA).

***In vitro* NIR-PIT and cell viability assay**

Cells were seeded onto 96-well plates at 5.0×10^3/well and incubated with Sib-IR700 or Pan-IR700 (20 μg/mL) for 6 hours at 37 ℃ at which point the cells were irradiated with NIR light at 0, 5, 10, 15, 20, and 25 J/cm^2. Cell viability was determined 12 hours

after NIR-PIT using the Cell Proliferation Kit II (XTT; Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's protocol.

CDX tumor model

Animals were maintained in specific pathogen-free conditions in the animal laboratory at Okayama University. BALB/c athymic (BALB/c-*nu/nu*) mice were purchased from Clea (Tokyo, Japan). Six-week-old female mice were used to establish a CDX tumor model. A total of 2.5×10^6 TE8 cells and 7.5×10^6 FEF3 cells or 2.5×10^6 TE8 cells alone were suspended in a 50% mixture of Matrigel (Corning, NY, USA) and inoculated subcutaneously into the right flank. The tumor xenografts were measured with an external caliper, and the tumor volume was calculated using the following formula: length × width^2 × 0.5. Resected tumors were processed as FFPE blocks or frozen tissue blocks with Tissue-Tek OCT Compound (Sakura Finetechnical Co., Tokyo, Japan).

PDX tumor model

A PDX tumor model was established as previously reported (37). In brief, adult BALB/c-*nu/nu* mice (6–10 weeks of age) were used as the host for xenografted tumors. Esophageal cancer tissue was obtained from donor patients' specimens. The tissue was

cut into ~3 mm diameter pieces using a scalpel or razor blade, with necrotic tissue removed if present. The fresh tumor fragments were transplanted subcutaneously (s.c.) into the right flank of anaesthetized mice. We defined this PDX model as F0 mice. The tumor volume growth was measured using the formula describe above, and mice were observed for a maximum of 70 days. After F0 PDX tumor volume reached at around 1 cm^3, tumor fragments were cut into 3 mm diameter pieces and transplanted subcutaneously into the right flank of anaesthetized mice, which were defined as F1 mice. The mice transplanted tumor fragment from F1 mice in the same way were defined as F2 mice.

***In vivo* NIR-PIT**

We randomized the mice used for both CDX and PDX models into a total of 8 groups (CDX: n=5/group; PDX; n=4/group) for the following treatments: (1) PBS treatment (Control); (2) Sib-IR700 NIR-PIT treatment (CAF PIT); (3) Pan-IR700 NIR treatment (Cancer PIT); (4) Sib-IR700 and Pan-IR700 NIR-PIT treatment (Dual PIT). In CDX models, we randomized the mice four days after inoculation, and 50 μg/body of Sib-IR700 or/and Pan-IR700 was injected intraperitoneally, Sib-IR700 and Pan-IR700 were simultaneously administrated in the dual-target group. Irradiation with NIR light

was performed 1 and 2 days after drug injection by a 690 nm continuous wave laser (BrixX 690; Omicron, Germany) at 50 J/cm^2 under isoflurane anesthesia. In PDX models, we randomized the mice after the tumor size reached around 150 mm^3. APC administration and NIR light irradiation were performed in the same manner as in the CDX models. The power density of 200 mW/cm^2 was measured with the optical power meter as described above. Each treatment was performed for two cycles in CDX models or three in PDX models.

Immunohistochemistry of CDX and PDX model tumor

To perform immunohistochemistry using αSMA antibody, we fixed the resected tumors in 10% paraformaldehyde and embedded in paraffin. Paraffin sections of 3μm thickness were dewaxed and soaked in 0.3% H_2O_2 in methanol at RT for 10 minutes to quench endogenous peroxidase activity. Then, we immunohistologically stained the paraffin sections with the αSMA (A2547, clone 1A4, Sigma-Aldrich) antibody as described previously. To perform immunohistochemistry using Sibrotuzumab, we followed the protocol previously reported (6). Briefly, we fixed the OCT-embedded sections (5 μm thick) of freshly frozen tumor tissues with 2% PFA at RT and soaked in 0.3% H_2O_2 in methanol at RT for 10 minutes to quench endogenous peroxidase activity.

After two PBS washes, we blocked the sections with protein block solution (Dako). After three PBS washes, we incubated the sections with Sibrotuzumab (1 µg/mL) for 1hour at RT in a humidified chamber. Following washing, we incubated the sections with rabbit anti-human IgG (AB2339624, 1:1000, Jackson Immune Research, PA, USA) for 1hour at RT in a humidified chamber and developed with the rabbit Dako EnVision+ System-HRP (DAB) kit as recommended. To perform immunohistochemistry for collagen 1, hyaluronic acid, Paraffin embedded tissue sections were deparaffinized and dipped into 0.3% H2O2 in methanol to prevent endogenous peroxidase for 10 minutes.

Antigen retrieval was performed by heating the sections over retrieval solution with microwave oven for 14 minutes. The sections were incubated with Anti-Collagen I antibody (Abcam, ab34710, Cambridge, UK) dissolved in Antibody diluent (Dako, S0809, Copenhagen, Denmark) 1:150 after the solution temperature became room temperature (RT). The sections were incubated with secondarily antibody dissolved in Antibody diluent (Dako). The sections were reacted with DAB Substrate/Chromogen buffer (Dako) for visualization and then were soaked into a bath of Hematoxylin for counterstaining. The sections were dehydrated and sealed on cover glass. For Hyaluronic acid (HA), biotinylated Hyaluronan binding protein (HABP) (EMD Millipore Corp., 385911, Billerica, MA, USA) was used to detect HA. Based on the

protocol above, the sections were blocked by Avidin/Biotin Blocking Kit (Nichirei Biosciences Inc, code 415041, Tokyo, Japan) after antigen retrieval. The sections were incubated with HABP dissolved in Antibody diluent (Dako) 1:200 for 1 hour. The sections were reacted with HRP-conjugated Streptavidin Kit (Nichirei Biosciences Inc, code 426062) to label peroxidase to streptavidin. It followed the same protocol above from visualization.

Statistical analysis

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics (38). We used the Mann-Whitney test or Student's t-test for two-group comparisons of in vitro and in vivo experiments; for multiple group comparisons, we used analysis of variance with Tukey's test. We evaluated the correlation of FAP expression with clinicopathological variables using Pearson's chi-squared test, Fisher's exact test, or Student's t-test, as appropriate. Overall survival (OS) and disease-free survival (DFS) were calculated using the Kaplan-Meier method and used the log-rank test to compare

subgroups. Hazard ratios (HRs) and 95% confidence intervals (CIs) for clinical variables were calculated using Cox proportional hazards regression in the univariate and multivariate analyses. Statistical significance was set at $p < 0.05$.

Data availability

Raw data for this study were generated at Okayama University. Derived data supporting the findings of this study are available from the corresponding author upon request.

Results

High expression of FAP in tumor stroma demonstrated poor prognosis in esophageal cancer

To explore the relationship of the expression of FAP and outcomes in esophageal cancer tissue, immunohistochemistry was performed for surgically resected specimens. Surgically resected specimens were stained with H-E to confirm the location and edge of the esophageal tumors. The intra-tumor tissue was then evaluated for FAP expression, and the FAP area index in all cells was calculated (Fig. 1A). The median FAP area index was found to be 6.07% (Fig. 1B), and based on this value, clinicopathological factors

were compared between the FAP high and FAP low groups (Supplementary Table 1). The FAP high group showed significantly deeper tumor invasion and lymph node metastasis compared to the FAP low group. In prognostic outcomes, the FAP high group had significantly shorter DFS and OS (Fig. 1C and D). Multivariate analysis revealed that FAP expression was an independent prognostic factor for DFS (HR = 2.12, 95% CI = 1.22-3.70; $p < 0.01$) and OS (HR = 1.79, 95% CI = 1.02-3.12; $p < 0.05$; Supplementary Table 2). These findings indicate that high FAP expression in intra-tumoral tissues is strongly associated with poor prognosis in esophageal cancer. To validate our findings in an independent cohort, we conducted an analysis of OS and PFS using RNA sequencing data from esophageal cancer cases in The Cancer Genome Atlas (TCGA). However, in contrast to the results based on IHC, we did not identify a significant correlation between the presence of FAP-positive CAFs and the long-term prognosis in esophageal cancer patients. (Supplementary Figure S2).

CAFs in esophageal cancers overexpress FAP and αSMA

To examine the expression of αSMA and FAP in CAFs, normal fibroblasts (FEF3 and WI38) were activated with conditioned medium from esophageal cancer cells (TE8), resulting in the generation of CAF-FEF3 and CAF-WI38, respectively. Initially, FAP

expression was measured as an indicator of the activation level in normal fibroblasts, CAFs, and cancer cells via flow cytometry (Fig. 2A). FAP was highly expressed in CAF-FEF3 and CAF-WI38 cells, whereas FEF3, WI38, and TE8 showed minimal or no expression. Western blot analysis also revealed overexpression of FAP and αSMA in CAF-FEF3 and CAF-WI38 cells compared to cancer cells and normal fibroblasts (Fig. 2B). To evaluate the in vivo impact of CAFs, a tumor consisting of TE8 cells alone and a co-inoculated tumor consisting of TE8 cells and FEF3 cells were established in BALB/c-*nu/nu* mice, and tumor growth was compared (Fig. 2C). The TE8+FEF3 co-inoculated group displayed a significantly higher tumor volume than the TE8 group. The status of αSMA expression was identified using immunohistochemistry (Fig. 2D), and the area index of αSMA expression was calculated to quantify the existence of CAFs. In the TE8+ FEF3 co-inoculated group, the value was significantly higher than that in the TE8 group (Fig. 2E). These findings suggest that normal fibroblasts were activated in response to stimulation by cancer cells, and the TE8 and FEF3 co-inoculated tumor can simulate a CAF-rich tumor model.

NIR-PIT using Sib-IR700 leads to the cell death of CAFs in vitro

To verify the successful synthesis of Sib-IR700, SDS-page was used to demonstrate

that the conjugated and unconjugated antibody had nearly equivalent molecular weights, and the Sib-IR700 exhibited an intense 700 nm fluorescence (Fig. 3A). The absorbance spectrum also confirmed the presence of IR700 in purified Sib-IR700, as evidenced by 690 nm peak (Supplementary Fig. S2), indicating successful conjugation between IR700 and the antibody. Subsequently, we evaluated the specificity of Sib-IR700 binding to CAFs. Consistent with FAP expression, Sib-IR700 bound to CAF-FEF3 and CAF-WI38 but not to FEF3 and WI38 cells (Fig.2A and Fig.3B). This signal was neutralized by excess Sibrotuzumab, suggesting that the affinity of Sib-IR700 was maintained. To assess the selectivity and efficacy of FAP-targeted NIR-PIT utilizing Sib-IR700 *in vitro*, we performed immunofluorescent microscopy, which revealed that Sib-IR700 bound to FAP on CAF-FEF3 and CAF-WI38 cells prior to NIR-PIT (Fig. 3C). Immediately following NIR irradiation, cellular swelling and bleb formation were observed in DIC images, and PI staining showed damaged CAFs. Conversely, no changes or damages were observed in FEF3 or WI38 cells. Additionally, a cell viability assay was performed to quantify the effect of NIR-PIT (Fig. 3D). The efficacy of NIR-PIT increased in a NIR light-dose dependent manner in CAF-FEF3 and CAF-WI38 cells, with 90 % reduction observed in CAF-FEF3 cells and 97% in CAF-WI38 cells after NIR-PIT with 25 J/cm^2 of NIR light irradiation. In contrast, NIR-PIT had little

cytotoxic effect on FEF3 and WI38 cells. These results demonstrate that FAP-targeted NIR-PIT utilizing Sib-IR700 can selectively destroy CAFs activated by cancer cells.

NIR-PIT using Sib-IR700 inhibited tumor growth in a CDX model

To evaluate the effect of FAP-targeted NIR-PIT using the Sib-IR700 *in vivo* model, we established co-engrafted tumors with FEF3 cells and TE8 cells expressing EGFR as previously described (24). According to the report, 61.4% of esophageal cancer cases tested positive for EGFR. Furthermore, the prognosis for cases that were double-positive for FAP and EGFR was notably and significantly poorer. First, we have verified the expression of EGFR in TE8 cells and the effective destruction of TE8 cells through cancer cell-targeted NIR-PIT using Panitumumab (Supplementary figure S3). In order to target CAFs deep within the tumor, our protocol was developed based on the findings of previous studies (24). It involves the administration of a sufficient dose of APCs and the application of two NIR irradiations. The co-engrafted tumor-bearing mice were randomized and treated with the following: a) no treatment (Control), b) NIR-PIT utilizing Sib-IR700 (CAF PIT), c) NIR-PIT utilizing Pan-IR700 (Cancer PIT), and d) NIR-PIT utilizing Pan-IR700 and Sib-IR700 (Dual PIT). The treatment regimen is presented in Figure 4A. The data from this study revealed significant suppression of

tumor volume in both the Cancer PIT and CAF PIT groups in comparison to the Control group, with the Dual PIT group exhibiting the most pronounced tumor inhibition (Fig. 4B). The harvested tumor weights on day 21 were also decreased in all PIT groups compared to the Control group (Fig. 4C and D). Additionally, no significant differences in body weight were observed among all groups, indicating no known systemic adverse events occurred (Fig. 4E). Irradiation with NIR light alone without prior APC administration or administration of the APC alone without NIR light did not affect tumor volume curves or harvested tumor weights (Supplementary Fig. S4 and S5). Immunohistochemical images revealed that the αSMA area index in the intratumoral tissues in the CAF PIT and Dual PIT groups were significantly lower than that in the Control and Cancer PIT groups (Fig. 4F and G). Our results suggest that FAP-targeted NIR-PIT using Sib-IR700 inhibited tumor progression and eliminated CAFs in the NIR light-irradiated area.

The expression of EGFR and TME in a PDX model mirrors that found in a primary tumor

To simulate the potential clinical application of NIR-PIT utilizing Sib-IR700, we established a PDX model from human esophageal cancer tissue. To ascertain whether

the PDX model faithfully represented the characteristics of primary tumors, each tumor was meticulously examined in resected specimens, alongside the CDX model. Initially, histological evaluation through HE staining revealed that the stromal structure present in primary tumors exhibited similar morphological features in the PDX F2 model, while in the CDX model, an interstitial structure was also observed, albeit with less intensity (Fig. 5). Subsequently, the same tissue sections underwent validation through IHC to determine if they preserved the stromal characteristics at the protein level. Positive staining for SMA and FAP, recognized markers of CAFs, was uniformly observed in all sections. In the PDX F2 model, the stromal formation closely mirrored that of primary tumors, with FAP and SMA staining consistent with the primary tumor site. Staining patterns for collagen and hyaluronic acid, key components of the stromal environment in addition to CAFs, closely resembled those observed in primary tumors in the PDX F2 model. In addition to stromal evaluation, examination of the tumor cells revealed EGFR expression in all sections (Supplementary Fig. S6). Furthermore, immunohistochemistry for PDX F2 tumor showed binding of Sibrotuzumab consistent with the stroma expressing αSMA in the intratumoral tissue (Supplementary Fig. S7). These findings collectively suggest that the PDX F2 model retains the properties of the stromal microenvironment and cancer cell characteristics observed in primary tumors.

NIR-PIT using Sib-IR700 inhibited tumor growth in vivo in a PDX model.

To verify the therapeutic effect of NIR-PIT using Sib-IR700 against a PDX model, we randomized tumor-bearing mice and compared the results with untreated PDX models. The treatment schedule for PDX models is shown in Figure 6A. Consistent with the outcomes observed in CDX models, both Cancer and CAF PIT interventions resulted in the suppression of tumor growth when contrasted with the Control group (Fig. 6B). The Dual PIT group showed the highest inhibition of tumor growth among all groups, with a significant decrease compared to the Control group. Similar results were observed for the tumor weights harvested on day 19 (Fig. 6C). However, there was no significant difference among CAF PIT, Cancer PIT, and Dual PIT groups perhaps because of the small number of cases (Fig. 6B and C). Regarding the assessment of CAFs, αSMA expression by immunohistochemistry in the CAF PIT and Dual PIT groups were clearly lower than in the other groups (Fig. 6D). Quantitative analysis of the αSMA area index demonstrated a significant decrease in CAF PIT and Dual PIT groups compared to other groups (Fig. 6E). Thus, these results suggest that FAP-targeted NIR-PIT using Sib-IR700 elicited tumor growth inhibition by depleting CAFs in PDX models, similar to CDX models.

Discussion

As previously delineated, NIR-PIT represents a pioneering approach to cancer treatment, with a particular focus on head and neck malignancies. Contemporary clinical practice has witnessed the integration of therapies aimed at cancer cells, prominently featuring EGFR as a principal target. This has swiftly become a standard practice, and there are substantial prospects for its further diversification in the future. However, it is of paramount importance to acknowledge that the benefits of cell-targeted therapies alone have not been universally observed in all cancer patients. This emphasizes the pressing need for ongoing advancements in cancer therapeutics. Consequently, our research has been steered toward CAFs, which exert a pivotal influence on the progression of cancer.

In our prior investigations, we achieved the successful development of FAP-targeted NIR-PIT through a murine tumor model, incorporating human cancer cells and fibroblasts. Our findings unequivocally established FAP-targeted NIR-PIT's capability to selectively eliminate CAFs, thereby manifesting a marked anti-tumor effect. Furthermore, our research provided compelling evidence for the synergistic effects

resulting from the combination of CAF-targeted NIR-PIT with cancer cell-targeted NIR-PIT. Considering these findings, CAF-targeted NIR-PIT emerges as an exceptionally promising therapeutic approach. In order to progress this therapy towards clinical adoption, we conducted experiments utilizing Sibrotumumab, an agent already approved for clinical use, in conjunction with a PDX model derived from human tumor tissue. Our results unequivocally demonstrated that the therapeutic effect closely mirrors that observed in our previous study. This research functions as a pivotal link between pre-clinical and clinical investigations of CAF-targeted NIR-PIT, marking a substantial advancement towards forthcoming clinical trials. The results achieved in this study closely align with the anticipated outcomes in the upcoming clinical trials, signifying significant progress in this field.

Although Sibrotuzumab was proven to be feasible and safe in a phase I clinical trial about 20 years ago, it did not demonstrate a suitable clinical response in a subsequent phase II clinical trial of patients with metastatic colorectal cancer. This is probably because treatments systemically targeting CAFs alone is likely to have minimal benefit while carrying significant systemic toxicities. Additionally, the number of CAFs within the treated tumor was not quantified in each case which could lead to a selection bias against a favorable outcome. Our study demonstrates that CAF-targeted treatments

depend on the amount of CAFs in the tumor, (Fig. 5 and 6) and therefore, careful patient selection is warranted with CAF-directed therapies. Furthermore, systemic depletion of FAP^+ cells induces severe cachexy, pancytopenia, and bone marrow toxicity in in vivo studies, suggesting that adverse events may be more frequent in cancer patients (34, 35). However, the application of FAP-targeted NIR-PIT using Sibrotuzumab does not depend on systemic blocking of FAP, but rather permits localized destruction of CAFs within the irradiated area of the tumor, thus, reducing the dosage of Sibrotuzumab required and resulting in fewer adverse events. Considering its safety, tolerability, and ability to target FAP, Sibrotuzumab is a highly suitable antibody to target FAP in the stroma of cancer tissue.

Our research has established a prognostic association between the presence of FAP-positive CAFs and the clinical outcomes of patients with esophageal cancer. This assessment was conducted through IHC analysis of resected tumor specimens. However, the absence of a similar correlation in the TCGA dataset highlights a potential discrepancy arising from the sole grouping of patients based on RNA expression levels. It is crucial to emphasize that, despite their relatively low numbers within the tumor microenvironment, the presence of FAP-positive CAFs holds significant immunological implications. Furthermore, the precise spatial distribution and location of these CAFs

within the tumor microenvironment are deemed critical factors. Hence, the evaluation of tumor-associated FAP-positive CAFs via IHC analysis using resected specimens is indispensable in fully comprehending their prognostic relevance.

Compared to other preclinical models such as CDX models or genetically engineered mouse models, PDX models better reflect the complex heterogeneity of human tumors, which often have unique molecular subtypes and cellular composition. Additionally, PDX models also retain the characteristics of the patient's original tumor, such as mutations and gene expression patterns. Therefore, as a preclinical study customized to individual patients, the PDX model represents an informative platform to investigate the efficacy and underlying mechanisms of NIR-PIT. In a previous study, we demonstrated the potential therapeutic efficacy of FAP-targeted NIR-PIT in a CDX model. To further evaluate the clinical feasibility of this approach, we established a PDX model and tested the antitumor effect of NIR-PIT using clinically available Sibrotuzumab. We observed significant suppression of tumor growth in a CAF-rich PDX model with FAP-targeted NIR-PIT using Sib-IR700. These results suggest that NIR-PIT with Sibrotuzumab could induce remodeling of the TME, leading to slowing of tumor growth.

EGFR-targeted NIR-PIT is in clinical use for inoperable or recurrent head and neck in Japan. There is a strong probability of expanding the indication to include esophageal

squamous cancer cell, which shares the same histological type and often overexpresses EGFR. The utilization of PDX derived from individuals with esophageal cancer is valuable in conducting preclinical studies which include both EGFR and FAP as targets, with the aim of extending the indication to encompass esophageal cancer patients in subsequent endeavors. In this study, we have evaluated the therapeutic efficacy of NIR-PIT targeting FAP-expressing CAFs induced by ESCC. Our prior research has confirmed the antitumor potential of NIR-PIT against FAP^{+}CAFs originating from adenocarcinomas. These findings suggest that the application of this approach can be extended to address CAFs in diverse cancer types beyond ESCC.

NIR-PIT can easily and simultaneously target two or more surface antigens. For instance, in cases where FAP-targeted NIR-PIT alone is not effective, the implementation of combinations of APCs can augment effectiveness. Altering the TME can reverse immune-permissive environments, enabling the immune system to act on the tumor, essentially an immunotherapeutic approach. Consequently, the deletion of CAFs, the predominant physical constituents of the TME in some cancers, represents a highly rational approach to amplifying tumor immunity. We showed the potential of this approach using a combination of anti-EGFR antibody and FAP-targeted NIR-PIT, and observed a synergistic effect in inhibiting tumor growth (Fig. 6). Although, in this study,

the combined therapy did not show a significant delay in tumor growth compared to each monotherapy, the strongest tumor growth inhibition and least individual differences were observed with the combined therapy. Furthermore, this approach has the potential to be applied not only to esophageal cancer but also to other types of CAF-containing cancers that also express EGFR. Moreover, NIR-PIT can target other cancer-specific antigens, making it a versatile treatment option.

Several clinical trials targeting FAP are ongoing. To improve anti-tumor immunity, FAP binding site is combined with IL-2v (NCT02627274), CD40 (NCT04857138) or CD137 (NCT04826003). NG-641 is a tumor-selective adenoviral vector that expresses a fibroblast activation protein-directed bi-specific T-cell activator antibody, hence anti-tumor immune response is enhanced (NCT05043714). These therapies are expected to shrink solid tumor, but there are concerns about various immune-related adverse events such as cytokine storm. In addition, FAP is expressed by not only CAFs but activated fibroblasts during wound healing in the skin, thus, therapy targeting FAP should be performed directly and specifically for CAFs in TME. Therefore, NIR-PIT using Sibrotuzumab is reasonable in therapies targeting FAP because of antibody specificity and local irradiation of NIR-light.

This study has some limitations. First, the evaluation of host tumor immune response

was not performed, as human-derived CDX and PDX models with athymic mice were used. Future experiments utilizing syngeneic mice and anti-mouse FAP antibodies may be needed to assess tumor immunity. In addition to immunological analysis, it is also important to analyze the mechanism of high efficacy of CAF PIT and to search for biomarkers to predict efficacy. Future exploratory studies of methods to predict FAP^+CAF rich tumors in tissue and liquid biopsies are warranted. Second, the efficacy of NIR-PIT was evaluated in xenograft models, and it may be more clinically relevant to evaluate its efficacy in orthotopic tumor models that better replicate the patients' TME (39, 40). Additionally, since NIR light was irradiated to subcutaneous tumors from outside the body in this study, adjustments may be necessary when irradiating NIR light in the lumen of the esophagus. Furthermore, it is important to acknowledge that this study has not been validated with an extensive number of PDX models. This limitation arises from the low success rate of PDX establishment and the variations introduced by preoperative treatments, making it challenging to generate PDX models from untreated resected esophageal cancer specimens. Thus, further investigation is needed to address these limitations.

In conclusion, our study demonstrates that FAP-targeted NIR-PIT using Sibrotuzumab selectively destroyed FAP^+ CAFs and suppressed tumor progression in CDX and PDX

models, which simulate the clinical setting of FAP-targeted NIR-PIT. Therefore, FAP-targeted NIR-PIT using Sibrotuzumab has the potential to be a novel cancer therapy for targeting and remodeling the TME in esophageal cancer.

Acknowledgement

We are grateful to Ms. Tomoko Sueishi and Ms. Tae Yamanishi for their technical assistance. This work was supported by JSPS KAKENHI Grant Numbers JP21K08754.

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References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209-49.

2. Huang FL, Yu SJ. Esophageal cancer: Risk factors, genetic association, and treatment. Asian J Surg. 2018;41(3):210-5.

3. Roma-Rodrigues C, Mendes R, Baptista PV, Fernandes AR. Targeting Tumor Microenvironment for Cancer Therapy. Int J Mol Sci. 2019;20(4).

4. Lin EW, Karakasheva TA, Hicks PD, Bass AJ, Rustgi AK. The tumor microenvironment in esophageal cancer. Oncogene. 2016;35(41):5337-49.

5. Noma K, Smalley KS, Lioni M, Naomoto Y, Tanaka N, El-Deiry W, et al. The essential role of fibroblasts in esophageal squamous cell carcinoma-induced angiogenesis. Gastroenterology. 2008;134(7):1981-93.

6. Kato T, Noma K, Ohara T, Kashima H, Katsura Y, Sato H, et al. Cancer-Associated Fibroblasts Affect Intratumoral CD8(+) and FoxP3(+) T Cells Via IL6 in the Tumor Microenvironment. Clin Cancer Res. 2018;24(19):4820-33.

7. Kashima H, Noma K, Ohara T, Kato T, Katsura Y, Komoto S, et al. Cancer-associated fibroblasts (CAFs) promote the lymph node metastasis of esophageal squamous cell carcinoma. Int J Cancer. 2019;144(4):828-40.

8. Katsube R, Noma K, Ohara T, Nishiwaki N, Kobayashi T, Komoto S, et al. Fibroblast activation protein targeted near infrared photoimmunotherapy (NIR PIT) overcomes therapeutic resistance in human esophageal cancer. Sci Rep. 2021;11(1):1693.

9. Nishiwaki N, Noma K, Ohara T, Kunitomo T, Kawasaki K, Akai M, et al. Overcoming cancer-associated fibroblast-induced immunosuppression by anti-interleukin-6 receptor antibody. Cancer immunology, immunotherapy : CII. 2023:1-16.

10. Watanabe S, Noma K, Ohara T, Kashima H, Sato H, Kato T, et al. Photoimmunotherapy for cancer-associated fibroblasts targeting fibroblast activation protein in human esophageal squamous cell carcinoma. Cancer biology & therapy. 2019:1-15.

11. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423-37.

12. Mao X, Xu J, Wang W, Liang C, Hua J, Liu J, et al. Crosstalk between

cancer-associated fibroblasts and immune cells in the tumor microenvironment: new findings and future perspectives. Mol Cancer. 2021;20(1):131.

13. Roberts EW, Deonarine A, Jones JO, Denton AE, Feig C, Lyons SK, et al. Depletion of stromal cells expressing fibroblast activation protein-α from skeletal muscle and bone marrow results in cachexia and anemia. J Exp Med. 2013;210(6):1137-51.

14. Glabman RA, Choyke PL, Sato N. Cancer-Associated Fibroblasts: Tumorigenicity and Targeting for Cancer Therapy. Cancers (Basel). 2022;14(16).

15. Puré E, Blomberg R. Pro-tumorigenic roles of fibroblast activation protein in cancer: back to the basics. Oncogene. 2018;37(32):4343-57.

16. Zi F, He J, He D, Li Y, Yang L, Cai Z. Fibroblast activation protein α in tumor microenvironment: recent progression and implications (review). Mol Med Rep. 2015;11(5):3203-11.

17. Duperret EK, Trautz A, Ammons D, Perales-Puchalt A, Wise MC, Yan J, et al. Alteration of the Tumor Stroma Using a Consensus DNA Vaccine Targeting Fibroblast Activation Protein (FAP) Synergizes with Antitumor Vaccine Therapy in Mice. Clin Cancer Res. 2018;24(5):1190-201.

18. Feig C, Jones JO, Kraman M, Wells RJ, Deonarine A, Chan DS, et al. Targeting

CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc Natl Acad Sci U S A. 2013;110(50):20212-7.

19. Tran E, Chinnasamy D, Yu Z, Morgan RA, Lee CC, Restifo NP, et al. Immune targeting of fibroblast activation protein triggers recognition of multipotent bone marrow stromal cells and cachexia. J Exp Med. 2013;210(6):1125-35.

20. Mitsunaga M, Ogawa M, Kosaka N, Rosenblum LT, Choyke PL, Kobayashi H. Cancer cell-selective in vivo near infrared photoimmunotherapy targeting specific membrane molecules. Nat Med. 2011;17(12):1685-91.

21. Kato T, Okada R, Goto Y, Furusawa A, Inagaki F, Wakiyama H, et al. Electron Donors Rather Than Reactive Oxygen Species Needed for Therapeutic Photochemical Reaction of Near-Infrared Photoimmunotherapy. ACS Pharmacol Transl Sci. 2021;4(5):1689-701.

22. Sato K, Ando K, Okuyama S, Moriguchi S, Ogura T, Totoki S, et al. Photoinduced Ligand Release from a Silicon Phthalocyanine Dye Conjugated with Monoclonal Antibodies: A Mechanism of Cancer Cell Cytotoxicity after Near-Infrared Photoimmunotherapy. ACS Cent Sci. 2018;4(11):1559-69.

23. Okada R, Furusawa A, Vermeer DW, Inagaki F, Wakiyama H, Kato T, et al.

Near-infrared photoimmunotherapy targeting human-EGFR in a mouse tumor model simulating current and future clinical trials. EBioMedicine. 2021;67:103345.

24. Sato H, Noma K, Ohara T, Kawasaki K, Akai M, Kobayashi T, et al. Dual-targeted near-infrared photoimmunotherapy for esophageal cancer and cancer-associated fibroblasts in the tumor microenvironment. Sci Rep. 2022;12(1):20152.

25. Cognetti DM, Johnson JM, Curry JM, Kochuparambil ST, McDonald D, Mott F, et al. Phase 1/2a, open-label, multicenter study of RM-1929 photoimmunotherapy in patients with locoregional, recurrent head and neck squamous cell carcinoma. Head Neck. 2021.

26. Sato K, Sato N, Xu B, Nakamura Y, Nagaya T, Choyke PL, et al. Spatially selective depletion of tumor-associated regulatory T cells with near-infrared photoimmunotherapy. Sci Transl Med. 2016;8(352):352ra110.

27. Okada R, Kato T, Furusawa A, Inagaki F, Wakiyama H, Choyke PL, et al. Local Depletion of Immune Checkpoint Ligand CTLA4 Expressing Cells in Tumor Beds Enhances Antitumor Host Immunity. Advanced Therapeutics. 2021;n/a(n/a):2000269.

28. Kato T, Fukushima H, Furusawa A, Okada R, Wakiyama H, Furumoto H, et al.

Selective depletion of polymorphonuclear myeloid derived suppressor cells in tumor beds with near infrared photoimmunotherapy enhances host immune response. Oncoimmunology. 2022;11(1):2152248.

29. Kato T, Furusawa A, Okada R, Inagaki F, Wakiyama H, Furumoto H, et al. Near-Infrared Photoimmunotherapy Targeting Podoplanin-Expressing Cancer Cells and Cancer-Associated Fibroblasts. Mol Cancer Ther. 2023;22(1):75-88.

30. Hidalgo M, Amant F, Biankin AV, Budinská E, Byrne AT, Caldas C, et al. Patient-derived xenograft models: an emerging platform for translational cancer research. Cancer Discov. 2014;4(9):998-1013.

31. Abdolahi S, Ghazvinian Z, Muhammadnejad S, Saleh M, Asadzadeh Aghdaei H, Baghaei K. Patient-derived xenograft (PDX) models, applications and challenges in cancer research. J Transl Med. 2022;20(1):206.

32. Okada S, Vaeteewoottacharn K, Kariya R. Application of Highly Immunocompromised Mice for the Establishment of Patient-Derived Xenograft (PDX) Models. Cells. 2019;8(8).

33. International Patent Number: WO99/57151, Example 3 and 4, CAS 216669-97-5 [cited 2024 Mar 27], Available from The World Intellectual Property Organization (WIPO):

https://patentscope2.wipo.int/search/en/detail.jsf?docId=WO1999057151&_cid=JP1-LUE4GA-97228-1

34. Scott AM, Wiseman G, Welt S, Adjei A, Lee FT, Hopkins W, et al. A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer. Clin Cancer Res. 2003;9(5):1639-47.

35. Hofheinz RD, al-Batran SE, Hartmann F, Hartung G, Jäger D, Renner C, et al. Stromal antigen targeting by a humanised monoclonal antibody: an early phase II trial of sibrotuzumab in patients with metastatic colorectal cancer. Onkologie. 2003;26(1):44-8.

36. Kato T, Okada R, Furusawa A, Wakiyama H, Furumoto H, Fukushima H, et al. Comparison of the Effectiveness of IgG Antibody versus F(ab')(2) Antibody Fragment in CTLA4-Targeted Near-Infrared Photoimmunotherapy. Molecular pharmaceutics. 2022.

37. Rivera M, Fichtner I, Wulf-Goldenberg A, Sers C, Merk J, Patone G, et al. Patient-derived xenograft (PDX) models of colorectal carcinoma (CRC) as a platform for chemosensitivity and biomarker analysis in personalized medicine. Neoplasia. 2021;23(1):21-35.

38. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for

medical statistics. Bone Marrow Transplant. 2013;48(3):452-8.

39. Hoffman RM. Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. Invest New Drugs. 1999;17(4):343-59.

40. Hoffman RM. Patient-derived orthotopic xenografts: better mimic of metastasis than subcutaneous xenografts. Nat Rev Cancer. 2015;15(8):451-2.

Figure Legend

Figure 1. Correlation between FAP expression and clinical outcome in esophageal cancer patients.

(A) Representative images showing H&E staining and immunohistological FAP expression in cases with high and low FAP levels. The dotted line indicates the tumor-stroma border. Scale bars: 1 mm and 200 μm (enlarged image). (B) Histogram illustrating the FAP area index for all cases, with the median value indicated by the black bar. (C, D) Survival analyses comparing the high FAP group to the low FAP group for (C) disease-free survival and (D) overall survival (n = 140, log-rank test and Cox regression hazard model; HR, hazard ratio; 95% CI, 95% confidence intervals).

Figure 2. FAP and αSMA overexpression in CAFs in vitro and in vivo.

(A) Flow cytometry histogram depicting FAP expression in fibroblasts, CAFs, and cancer cells. (B) Western blot analysis displaying the expression of FAP and αSMA in fibroblasts, CAFs, and cancer cells. (C) Tumor growth curves in xenograft models (n = 7, mean ± SD, Student's t-test; * $p < 0.05$). (D) Representative images of immunohistochemical staining for αSMA in harvested tumors. Scale bars: 100 μm and 50 μm (enlarged image). (E) Average number of αSMA area index in xenograft models (n = 7, Student's t-test; *$p < 0.05$).

Figure 3. NIR-PIT using Sibrotuzumab-IR700 in vitro.

(A) Validation of Sibrotuzumab-IR700 and Panitumumab-IR700 by SDS-PAGE (left: Colloidal Blue staining, right: 700 nm fluorescence). Each diluted antibody served as a control. (B) Flow cytometric analysis of Sibrotuzumab-IR700 binding to fibroblasts and CAFs. Histograms show representative results. (C) Microscopic images before and after NIR-PIT. Scale bars: 50 μm. (D) Cell viability of fibroblasts and CAFs induced by NIR-PIT, measured using metabolic activity by XTT assay (n = 5, mean ± SD, one-way ANOVA followed by Tukey test; *$p < 0.05$). In A, C, and D, the representative examples from four experiments were shown.

Figure 4. NIR-PIT using Sibrotuzumab-IR700 was effective in CDX model.

(A) In vivo therapy protocol. (B) Tumor growth curve in TE8+FEF3 tumors (n = 5, mean ± SD; one-way ANOVA followed by Tukey test; *$p < 0.05$; **$p < 0.01$). (C) Macroscopic images of harvested tumors on day 21. (D) Tumor weights among the four groups for TE4+FEF3 tumors (n = 5, mean ± SD; one-way ANOVA followed by Turkey's test; *$p < 0.05$; **$p < 0.01$). (E) Body weights for TE4+FEF3 tumors (n = 5, mean ± SD; one-way ANOVA followed by Turkey's test; N.S., not significant). (F) Representative images of immunohistochemical staining for αSMA in harvested tumors treated by NIR-PIT. Scale bars:1 mm and 200 μm (enlarged image). (G) Average number of αSMA area index in treated TE8+FEF3 xenograft models (n = 5, Student's t-test; *$p < 0.05$).

Figure 5. Immunohistochemistry for primary tumor, PDX, and CDX models.

Comparison of H.E. and immunohistological images (FAP, SMA, Masson's trichrome, Collagen I, and Hyaluronic acid) in a primary tumor, a PDX F2 tumor, and a CDX tumor. Scale bars: 200 μm.

Figure 6. NIR-PIT using Sibrotuzumab-IR700 was effective in PDX model.

(A) In vivo therapy protocol. (B) Tumor growth curve in PDX tumors (n = 5, mean ± SD; one-way ANOVA followed by Tukey test; *$p < 0.05$). (C) Tumor weights among the four groups for PDX tumors (n = 5, mean ± SD; one-way ANOVA followed by Turkey's test; *$p < 0.05$). (D) Representative images of immunohistochemical staining for αSMA in harvested tumors treated by NIR-PIT. Scale bars: 1 mm and 200 μm (enlarged image). (E) Average number of αSMA area index in treated PDX models (n = 5, Student's t-test; *$p < 0.05$).