Master of Science

Research on the development of antibody therapeutics to increase the survival rate of pancreatic cancer patients

췌장암 환자의 생존율을 높이기 위한

항체 치료제 개발 연구

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Research on the development of antibody therapeutics to increase the survival rate of pancreatic cancer patients

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Abstracts

Pancreatic cancer is difficult to be recognized, because there are no symptoms in its early stages. In most cases, after being diagnosed with pancreatic cancer, the survival rate is very low because of its metastasis to other organs. If antibodies that specifically bind to pancreatic cancer are found, it is premised that antibody-therapeutic conjugates can be created only for certain cancer cells and used for treatment. Therefore, the goal is to identify patient-derived antibodies, and create new customized antibody therapy through specific antigen-antibody binding of tumors.

An antibody (scFv) library is created using pDR-D1 (phagemid vector) from the blood obtained from 10 pancreatic cancer patients, and an antibody library is screened using the phage display technology to select antibody clones that specifically bind to the cancer cell. The selected scFv candidates were cloned into pDR-OriP-Fc1 (mammalian cassette vector) as an expression vector. HEK 293E cells were used to express scFv-Fc and tested for binding to pancreatic cancer cells through FACS.

As a result, we screened antibodies that strongly bind to PANC-1, a human pancreatic cancer cell line isolated from a pancreatic carcinoma of ductal cell origin. [38] In addition, it was confirmed that the selected antibodies also bound to breast cancer, lung cancer, colon cancer, and ovarian cancer cell lines.

The selected antibody is used for immunoprecipitation with pancreatic cancer cells to identify antigen through LC-MS/MS. After siRNA to knockdown of the candidate antigen, altered antibody binding was monitored by FACS. The difference in antigen-antibody binding after candidate gene knockdown was confirmed by FACS analysis. The most promising genes for antigens are MUC5AC, LARP7, DSP, SERPINA4, and GRPEL1.

Once the antigen for the selected antibody binds is confirmed, its antitumor effect will be examined in patient-derived heterogeneous transplant (PDX) model by antibody monotherapy or combinatory immunotherapy. Our study suggests that antibodies selected from the pancreatic cancer patient can be used as a therapeutic agent for the treatment of pancreatic cancer.

Keywords: Pancreatic cancer, phage display, Antibody library, immunotherapy, scFv, cell panning

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Introduction

Cancer is a disease that causes cells to grow abnormally to form masses, move to different locations in the body, and interfere with the functions of various organs and tissues necessary for life support, resulting in death. Pancreatic cancer accounts for about 3% of all cancers and about 7% of all cancer deaths in the United States.[2]

In 2021, the 5-year survival rate for pancreatic cancer was 10%, which was recorded lower than other cancers, and only when pancreatic cancer is identified at an early stage, the 5-year survival rate records at 39%. [2, 3] However, it is difficult to diagnose pancreatic cancer at an early stage because there are no recognizable symptoms, and when discovered, most patients have metastasized to other organs. [4, 5] Therefore, the difficulty of early pancreatic cancer screening means that early diagnosis can increase the survival rate.

There are surgical and anticancer treatments for pancreatic cancer. In general, surgical resection is the most effective way to remove cancer, but unlike general cancers, the pancreas itself is surrounded by various organs such as the duodenum, biliary tract, gallbladder, and spleen, making it quite difficult to remove the cancer. In addition, surgery is difficult because many patients are diagnosed after metastasis to areas other than the pancreas. Pancreatic cancer often reoccurs even after surgery, contributing to symptom relief and survival rate improvement through chemotherapy.

Pancreatic cancer anticancer drugs commonly used in Korea and abroad include FOLFIRINOX, Abraxane, and Gemcitabine. Typically, Gemcitabine has been used as a standard treatment for the past 20 years. However, chemotherapy is injected throughout the body, which can cause side effects on normal cells or cancer resistance to anticancer drugs, which can lead to more serious situations. And it is known that the presence of a BRCA mutation, one of the DNA damage recovery genes, responds well to FOLFIRINOX therapy. However, the BRCA mutation is about 5% of the world's patients, and it is difficult to expect an effect as an anticancer treatment due to the low frequency of pancreatic cancer patients in Korea.

For this reason, the most important problem in the development of anticancer drugs is to have the selectivity that specifically acts on cancer cells. As a result, the growth of the market is shifting from chemotherapy to targeted therapy with few side effects, and the treatment trends are shifting to personalized treatment approach.

As mentioned earlier, targeted treatments have been developed and used to minimize side effects. To improve the side effects of chemotherapy, there is a treatment using Cetuximab-targeted anticancer drugs that selectively

inhibit epidermal growth factor receptors (EGFR), which 90% of pancreatic cancers overexpressed. However, there is a limitation in that more than 95% of pancreatic cancer is modified in the KRAS gene, which is the EGFR down the signal pathway, and targeted anticancer drugs do not work. [6,39]

Recently, immuno-cancer drugs that suppress PD-1 have been widely used in various cancers, including lung cancer, but chemotherapy is currently the best treatment because effective immuno-cancer drugs have not yet been developed in pancreatic cancer.

This requires the discovery of customized cancer treatment targets and antibodies that can be applied to each patient, which can greatly contribute to the development of customized targeted treatment and treatment strategies for patients with refractory cancer resistance.

To overcome the limitations of existing pancreatic cancer treatments, this study was conducted to find antibodies that specifically bind to pancreatic cancer and develop antibody-drug conjugates to induce immunological treatment effects. This is because cancer immunotherapy is also based on inducing a person's immune system to attack tumor cells. Therefore, if an antibody that binds to pancreatic cancer is found by utilizing the various immune systems of the pancreatic cancer patient, it can be used for treatment and diagnosis to increase the survival rate. [8]

Since cell surface antigens are important targets in the development of therapeutic agents, a library derived from pancreatic cancer patients was created and utilized in phage display technology to find antibodies that specifically bind to pancreatic cancer. Phage display technology has led to the formation of antibody libraries and has provided techniques for fast and efficient search of these libraries.[7] Personalized therapeutic approaches, particularly in oncology, depend on the identification of new, unique, and functional targets that phage display, through its various declinations, can certainly provide. [8] Furthermore, the procedure gives chances to target novel epitope space created by disease-related overexpression or modification of cell surface proteins.[13]

The library we used here is in the form of scFv. The scFvs are produced by fusing the variable regions of the antibody’s heavy and light chains, creating a much smaller protein with unaltered specificity. [10] Because of its small size and relative ease of production, scFvs are a promising diagnostic and therapeutic reagents for protein misfolded diseases. [2, 10] The cloned scFv is designed to fuse to human Fcγ1 when expressed in mammalian cells. pDR-OriP-Fc1 includes OriP sequences which allow enhanced and prolonged protein expression in HEK 293E cells. [1] It is expressed as a scFv-Fc form.

In this way, we panned three types of pancreatic cancer cells using the human scFv library to obtain several candidate antibodies. And it was confirmed that two antibodies with specific bonds were also bound to other pancreatic cancer cells.

Materials and Methods

Antibody library construction

A library was prepared using the blood (10 mL of each blood) of 10 pancreatic cancer patients (The Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, China). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by standard density-gradient centrifugation using Ficoll® Paque Plus (Cytiva, Cat. No: 17-1440-02) and lysis buffer (BD, Cat. No: 555899). The manufacturer's instructions were followed. RNA was extracted from PBMCs by Tri-RNA reagent (FAVORGEN, Cat No. FATRR 001) and reverse-transcribed to cDNA with reverse transcription reagent kits according to manufacturer's instructions. [36]

Briefly, the cDNAs encoding human heavy and kappa chain variable regions (VH and VK) were amplified by PCR and the amplified VH and VL containing the BbsI site were linked by ligation. [14] The combined scFv repertoire was subjected to expansion PCR with primers containing the SfiI site. [13] Then amplified scFv inserts and pDR-D1 which is a phagemid vector for cloning and phage display of the scfv were digested with SfiI and ligated. The ligated constructs were electroporated into E. coli ER2738 cells. This experiment was conducted at KRIBB [13]. Bacteriophages displaying the scFv repertoire were rescued by the infection of the transformed cells with VCSM13 helper phage (Stratagene, La Jolla, CA) [12, 13]. The rescued phage library was used for cell panning.

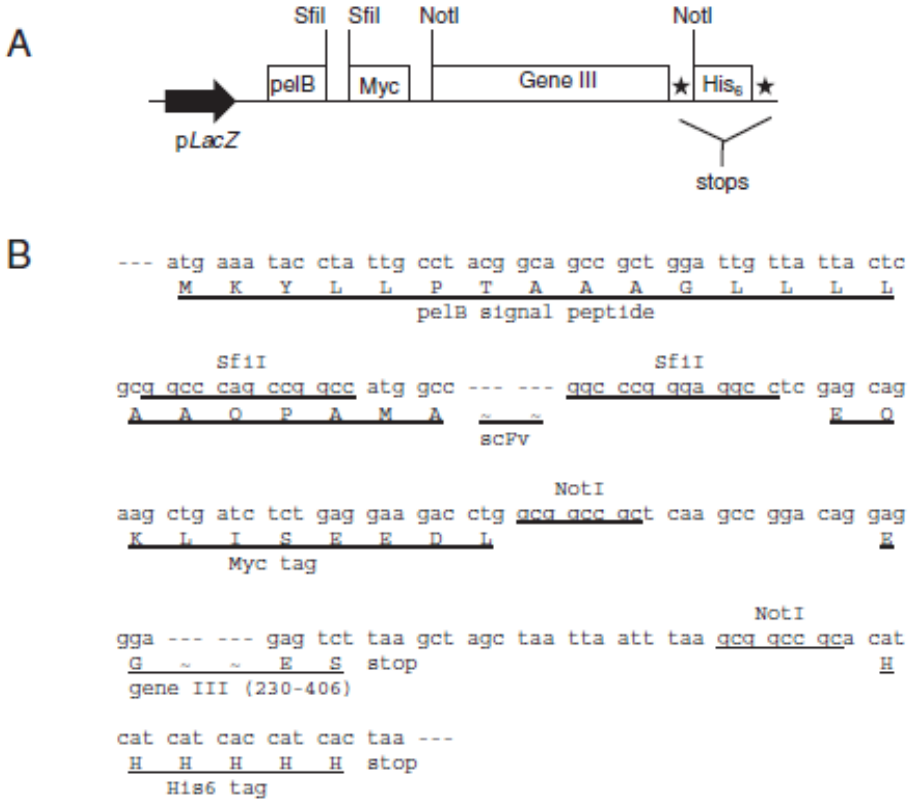


Figure 1. Schematic representation (A) and sequences (B) of major components of phagemid vector, pDR-D1 for phage display. (provided by Yoon H, Song JM, Ryu CJ, Kim YG, Lee EK, Kang S, Kim SJ. An efficient strategy for cell-based antibody library selection using an integrated vector system. BMC Biotechnol. 2012 Sep 18;12:62. doi: 10.1186/1472-6750-12-62. PMID: 22989299; PMCID: PMC3505469.)

The vector is derived from pComb3H with some modifications which result in SfiI-SfiI cassette sequences for scFv cloning followed by pelB signal sequences and gene III sequences removable by NotI-NotI between two tags, Myc and hexahistidine (His6) tags. The expression unit is under the control of the LacZ promoter (pLacZ).[13]

Cell cultures

The preparation methods for Cell panning were as follows: Pancreatic cell line cells(PANC-1) were grown in Dubecco's modified Eagle's Medium (DMEM, Cytiva, Cat No. SH30243.01) supplemented with 10% fetal bovine serum(FBS, GE Healthcare Life sciences, Cat No. SH30919.03), 1% penicillin/streptomycin(P/S, Gibco, Cat No. 15140-122). And pancreatic primary cancer cells (110621, 115026, 17884, 19224) were grown in RPMI-1640 (Cytiva, Cat No. SH30027.01) medium containing 5% FBS, 1% P/S, 4 μg/mL hydrocortisone (Sigma-Aldrich, Cat. H0888), 4 μg/mL transferrin (Sigma-Aldrich, Cat. T8158), 20 ng/mL EGF (Gibco, Cat. PHG0311). The PDC cell, which is a primary cell, was manufactured and stored according to the method of this laboratory. [40] HPDE cell was maintained in keratinocyte serum-free medium (Gibco, Cat. 10724-011) supplemented with 1% penicillin/streptomycin(P/S), supplements (bovine pituitary extract (Gibco, Cat. 13028-014), EGF (Gibco, Cat.10450-013)).

The preparation methods for scFv-Fc expression were as follows: HEK 293E cells (CRL-10852, ATCC) in DMEM containing 10% FBS, 1% P/S.

The preparation method of cells used for FACS is as follows: Pancreatic cancer cell line cells (PANC-1, PKCY, Pan02) were grown in DMEM supplemented with 10% FBS and 1% P/S. Human pancreatic primary cancer cells (110621, 115026, 17884, 19224) were grown in RPMI-1640 medium containing 5% FBS, 1% P/S, 4 μg/mL hydrocortisone, 4 μg/mL transferrin, 20 ng/mL EGF.

Breast cancer cell line cells (MDA-MB-231, MCF7) were grown in DMEM supplemented with 10% FBS and 1% P/S. Human breast primary cancer cell (050786) was grown in RPMI-1640 medium containing 5% FBS, 1% P/S, 4 μg/mL hydrocortisone, 4 μg/mL transferrin, 20 ng/mL EGF. Breast normal cell line cell (MCF10A) was grown in RPMI-1640 medium containing 10% FBS, 1% P/S, 4 μg/mL hydrocortisone, 4 μg/mL transferrin, 20 ng/mL EGF.

Ovarian cancer cell line cell (SK-OV-3) was grown in RPMI-1640 medium containing 10% FBS, 1% P/S.

Colorectal cancer cell line cell (HCT-116) was grown in RPMI-1640 medium containing 10% FBS, 1% P/S.

Lung cancer cell line cell (H460) was grown in RPMI-1640 medium containing 10% FBS, 1% P/S.

Normal human cell line cells (HEK 293E, HEK 293T Lenti-X) were grown in DMEM supplemented with 10% FBS and 1% P/S.

All the cells were cultured below humidified 5% s(*v/v*) CO2 incubator at 37 °C and 0.25% Trypsin (Gibco, Cat No. 15090-046) was used to remove the cells from the dish.

Cell panning

Four types of cells (HPDE, PANC-1, 110621, 115026) were prepared which were plated at >90% confluency in 6-well plates. Four kinds of cells confluency were prepared to be over 90%. Block HPDE cells using blocking solution (4% milk/PBS with Ca+ and Mg+) at 37°C for 1 hour. Before phage incubation, we use 4 ml PBS to wash blocked cells, 1 min at RT 3 times. Add 1 ml diluted phage (final conc. 10^{12} cfu/ml) to HPDE cells plated in 6-well plates for incubation, gently rocked at 4°C for 2hr. After one hour of incubation in HPDE cells, blocking cancer cells (110621, 115026, PANC-1) using blocking solution at 37°C for 1 hour. Before phage incubation to cancer cells, we use 4 ml PBS to wash blocked cells, 1 min at RT 3 times. The phage supernatant reacted with HPDE cells was transferred to a cancer cell plate and reacted at 4°C for 4 hours. Wash 5 times with 2ml PBS (w/o Ca+ and Mg+) for 1 min at room temperature, gently shaking by hand. Add 0.6 ml cold EDTA/PBS into one 6-well, stay 4 min on ice. Using cell lifter to harvest cells and wash once with 0.4 ml cold PBS. Transfer cells to 2 ml low binding tube (Axygen, cat. No. MCT-150-L-C, MCT-200-L-C), centrifuge (2000 rpm, 3min, 4°C. Wash cell twice with 5%FBS/PBS. Thereafter, the phage is eluted using 100 ul 0.1M Glycine-HCl (pH 2.2) Add 10 uL eluate to 190 ul ER2537 cell culture medium (OD600=0.7~1) and hold for 15 min at room temperature, then incubated overnight at 37 °C in an LB Agar plate containing ampicillin antibiotic after spreading. To amplify phages for the next panning round, supernatants were added to 1 mL of ER2537 of E. coli (OD600 = 0.7~1) grown in SB broth and incubated at room temperature for 15 minutes. Add 4 ml of pre-warmed SB and shake at 37 °C for 1 h at 300 rpm. The suspension was added to 50 ml SB broth with ampicillin antibiotic, Add 12.5 ul VCSM13 helper phage (2.5 x 10^{11} pfu), incubation at 37°C for 60 min at 80 rpm. Then add 35 ul kanamycin antibiotic, shaking overnight (10hrs) incubation at 250 rpm and 30°C. The culture medium was centrifuged (5000g, 20min, 4°C), the supernatant was transferred to a new tube, 5x PEG was added, and the reaction was performed on ice for 2 hours to precipitate phages. The reaction solution was centrifuged (10,000g, 30 min, 4°C), and the pellet was suspended in 3 ml cold PBS(w/o), divided into 1.5 mL microtube, and centrifuged (10,000g, 10 min, 4°C) again. After sterile filtration of the supernatant with a 0.2 um syringe filter, the supernatant was separated into three 1.5 ml tubes. 5x PEG was added to the supernatant, gently mixed, and incubated at 0 °C for 1.5 hours. After

centrifugation (15,000g, 30 min, 4°C) and removal of the supernatant, the pellet was resuspended in sterile PBS containing 0.02% sodium azide and stored at 4°C until used for the next panning.

Cell panning analysis

Analysis was performed after the second round of panning. 10 uL of eluted phage was infected with 190 uL ER2537 cell culture medium (OD600=0.7~1) at room temperature for 15 minutes, and the titer was confirmed by spreading on an LB ager plate containing ampicillin antibiotic. And then 100 colonies were randomly selected and cultured overnight in LB broth containing ampicillin, and DNA was extracted using a DNA extraction kit (GeneAll, Cat No. 101-102). Referring to Figure 2, the primer was designed to check the sequence bound in the vector. (Supplement table 1) To confirm the sequence of this, sequencing services were performed by Macrogen Inc.. Matching sequences were grouped and analyzed. (Using phagemid primer: F- 5'-GCA ATT AAT GTG AGT TAG CTC ACT-3', R- 5'-CCC TTA TTA GCG TTT GCC ATC TT-3')

To align the scFv sequence, 'Multiple Sequence Alignment by CLUSTALW' (https://www.genome.jp/tools-bin/clustalw) and 'Multiple sequence alignment by Florence Corpet' (http://multalin.toulouse.inra.fr/multalin/) site was used for analysis.

Cloning for expression of scFv-Fc

A mammalian expression vector, pDR-OriP-Fc1(Figure 2), was used to rapidly convert the phage display scFv to the Fc fusion form. The vector is derived from pComb3H with some modifications which result in SfiI-SfiI cassette sequences for scFv cloning followed by pelB signal sequences and gene III sequences removable by NotI-NotI between two tags, Myc and hexahistidine (His6) tags. The expression unit is under the control of the LacZ promoter (pLacZ). [13] The phagemid DNA and pDR-OriP-Fc1 were cloned by cutting with sfiI restriction enzyme (Enzynomics, Cat No. R033S). First, phagemid DNA and pDR-Orip-Fc1 were reacted with SfiI restriction enzyme (1 hour, 50°C) and they were electrophoresed in 1% and 0.8% agarose gels to confirm the band size. Then insert and vector were extracted using a gel extraction kit. (FAVORGEN, Cat No. FAGCK001-1). Second, the extracted insert and vector were combined using a ligation reagent (Enzynomics, Cat No. M001S) at room temperature for 1 hour. Third, after transformation into competent cells (DH5α), spread on LB agar plate containing ampicillin. Finally, to check the cloning result, referring to Figure 2, the primer was designed and

proceeded to check the sequence about cloned products. (Supplement table 1) Colony PCR {95℃ 3min, 29cycles (95℃ 30sec, 55℃ 40sec, 72℃ 1min) using primers (pDR-OriP-Fc1_F, pDR-OriP-Fc1_R, 3,43,54_F, 6,54_F, 16_F), 72 ° C. 5 min} was carried out. The size was confirmed through agarose gel electrophoresis, and only the clones that were confirmed to have the expected size were analyzed for sequence analysis. It has been confirmed that the alignment of this sequence result matches the scFv sequence before cloning.

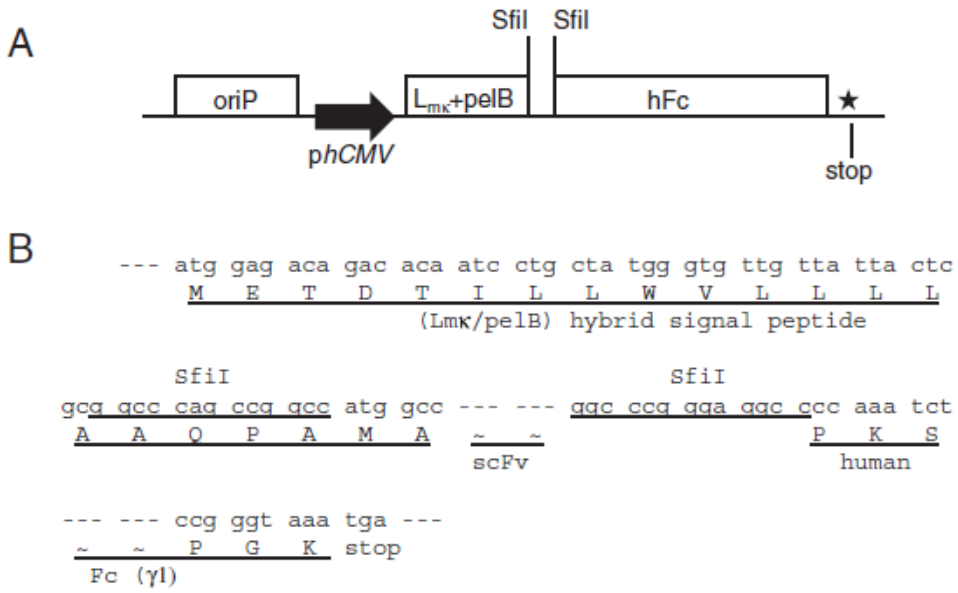


Figure 2. Schematic representation (A) and sequences (B) of major components of mammalian cassette vector, pDR-OriP-Fc1 for transient expression of scFv-Fc. (provided by Yoon H, Song JM, Ryu CJ, Kim YG, Lee EK, Kang S, Kim SJ. An efficient strategy for cell-based antibody library selection using an integrated vector system. BMC Biotechnol. 2012 Sep 18;12:62. doi: 10.1186/1472-6750-12-62. PMID: 22989299; PMCID: PMC3505469.)

The vector is derived from pcDNA3.1 with some modifications which result in the same SfiI-SfiI cassette sequences as pDR-D1 for scFv cloning followed by hybrid signal sequences composed of mouse kappa leader (Lmκ) and pelB signal sequences. Human Fcγ1 (hFc) sequences and hinge region are followed by the cloning site to allow in-frame fusion of scFv and hFc. The scFv-Fc expression unit is under the control of the human cytomegalovirus promoter (phCMV). oriP sequences are also included to mediate episomal amplification and maintenance of the transfected episomal DNA in 293E cells.[13]

Expression of scFv-Fc and purification

The resulting scFv-Fc expression plasmid was introduced into HEK 293E cells (CRL-10852, ATCC) using Lipofectamine™ 3000 Transfection Reagent (Thermofisher, Cat No. L3000015). The transfected cells were grown in DMEM containing 10% FBS and subsequently, the media were changed to serum-free media, and the culture supernatant was harvested once a day for 3 days. [13]

To purify scFv-Fc, 500uL (slurry 1:1) of Protein G beads (GE Healthcare, Cat No. 17-0618-01) washed with binding buffer (20mM sodium phosphate, pH 7.0) were added to the supernatant harvested for 3 days, and a rotator machine was incubated (4℃, 30rpm, 2hr). After centrifugation (700g, 30sec), remove the supernatant, add 10mL of PBS, wash, and centrifuge (700g, 30sec) again. After removing the supernatant, 1mL of PBS was put in the bead, transferred to a 2mL tube, centrifuged (1000rpm, 30sec), and the washing process was repeated 3 times. Add 450uL Elution buffer (0.1M glycine-HCl, pH 2.7), invert for 5 minutes, centrifuge (5000rpm, 2min), put 400uL of the supernatant into a new microtube, and add 30uL neutralizing buffer (1M Tris-HCl, pH 9.0) for inactivation. and stored refrigerated. All procedures were following the manufacturer's instructions.

Immunoprecipitation and immunoblotting

To confirm the size of the purified Ab through Western blot, it was loaded on 10% SDS PAGE (180V, 1.2hr) and transferred to an NC membrane (Cytiva, Cat No. 10600003) (80V, 2hr). After blocking with 3% BSA (in TBS-T) (Mpbio, Cat No. 160069) for 1 hour, goat anti-human IgG Fc antibody (HRP) (Life technologies, Cat No. A18817) was diluted 1:2000 in 3% BSA (in TBS-T) and reacted overnight in the refrigerator using the rocker. Using Tris-buffered saline (TBS-T) (20 mM Tris/HCl, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100), the membrane was washed 3 times for 15 minutes each on a rocker. Then The size of the purified Ab was confirmed using ECL (Cytiva, Cat No. RPN2106) or WestGlow™ Pico Plus (Biomax, Cat No. BWP0200).

For immunoprecipitation, pancreatic cancer cells were prepared at a confluent concentration of 80% or more in 100π cell culture dishes, respectively, and washed twice with PBS. 1.5 mL of EB (PI + PPI) buffer was added and incubated on ice for 10 minutes. Cells were harvested in a tube using a cell scraper, centrifuged (4℃, 12,000rpm, 5min), and the supernatant was divided into three tubes of 450uL each in a 1.5mL microtube, and a small amount

of 50uL was stored to confirm the input sample. Two representatives purified Abs and human IgG selected for each pancreatic cancer cell were put into each tube, 1ug each, mixed well, and incubated (4℃, 30rpm, 2hr). Protein G beads washed with EB (PI + PPI) were added to the reaction solutions by 20 uL, followed by incubation (4° C., 30 rpm, 1 hr) and centrifugation (5,000 rpm, 20 sec). The supernatant was removed, and washing was performed twice with 400uL EB (PI+PPI) buffer using a cut tip. After removing the supernatant from the washed beads as much as possible, 30uL of 1XSB was added, 2X SB was mixed 1:1 with the stored input sample, and protein boiling (95℃, 5min) was performed. This was centrifuged and 10 uL of the supernatant was loaded on a 10% SDS PAGE gel.

To check the antibody size, the gel was put into Coomassie blue (Bio-Rad, Cat No. 1610786) and stained (~30min, low speed) on a rocker. After removing Coomassie blue, water was added and washed on a rocker until the band was visible.

For LC-MS/MS, sliver staining was performed using a Pierce Silver Stain kit (Thermo Fisher, Cat No. 24612). The SDS PAGE gel was washed twice with sterile water (5min, 20rpm), 15mL of Fix buffer was added, and reacted twice (5min, 20rpm) to fix. Washed twice with 15mL of wash buffer (5min, 20rpm) and washed twice with sterile water (5min, 20rpm). 12.5 mL of Sensitizer Working Solution was added, reacted for exactly 1 minute, and washed twice (1 min, 20 rpm) with sterile water. 12.5 mL of Stain Working solution was added, reacted at room temperature for 30 min, and washed twice (20 sec) with sterile water. Add 12.5 mL of Developer Working solution and shake it well by hand to see if the band comes out. Then when the band was confirmed, the solution was discarded and a Stop solution was immediately added to stop. After incubation for 10 minutes, after washing with sterile water, LC-MS/MS analysis was requested to the Asan Life Science Research Institute's Protein Analysis Core Lab.

Migration assay and proliferation assay

To confirm proliferation, PANC-1 cells were seeded on 96 well plates by containing 0.1~1ug antibodies to be included in 1,000 cells/wells in a 100 μL culture medium. And under the same conditions, PDC cells(17884) were seeded 2,000 cells. It was changed to a 100 μL culture medium containing 1ug antibody every 24 hours.

HPDE cells and PDC cells(115026,19224) were seeded on 96 well plates by containing 0.1~0.5ug antibodies to be included in 1,500 cells/wells in a 100 μL culture medium. H460 cells were seeded on 96 well plates by containing 0.1~0.5ug antibodies to be included in 1,000 cells/wells in a 100 μL culture medium.

This process continued for up to 72 hours.

It was measured with a plate reader (450nm) at intervals of 30 minutes to 1 hour from 0.5 to 3 hr using EZ-CYTOX (DoGen, Cat No. EZ-1000). Cell viability according to the growth of 24, 48, 72hr was measured. The data was calculated as (mean Human IgG, antibodies treated A450 – mean blank) / (mean (Human IgG treated A450 -mean blank)) x 100 and analyzed. The IC_{50} value was then determined using the Graphed Prism software.

To confirm migration, 10 uL of 10% poly L (Sigma Aldrich, Cat No. P8920) was spread to the bottom of the trans-well (Costar, Cat No. 3422) and coated. Each antibody 1~2ug and $5x10^4$ cells were put in a 100uL serum-free medium and transferred to the coated trans-well. 500uL of medium containing each cell cultured serum was placed in a 24-well plate, and the trans-well was carefully transferred. All the cells were cultured below humidified 5% s(*v/v*) CO2 incubator at 37 °C for 24~48hr.

Methanol, Hematoxylin, and Eosin Y were dispensed into a new 24-well plate by 600ul each, and the trans-wells were placed in the order of Methanol (1min) and Hematoxylin (5min). The trans-well was placed in sterile water, washed from side to side, and water on the side of the trans-well was removed. After treatment with Eosin Y (1min) and washing with sterile water, the inside of the trans-well was well wiped with a blunt cotton swab, and the results were checked the next day.

Antibody binding confirmation through FACS

To confirm the binding of the purified antibody to cells, the cells were washed with FACS buffer (3% FBS in PBS) and then prepared by counting $5x10^5$ cells. Human IgG (cloud-clone, Cat No. NPA544Hu01) was used as a negative control (NC), Cetuximab was used as a positive control (PC), and the primary antibody (NC, PC, purified Ab) was 1~2ug in 100ul of FACS buffer. It reacted on ice for 2 hours.

After washing 3 times with FACS buffer, the secondary antibody, Fluorescein (FITC) AffiniPure Goat Anti-Human IgG Fcγ fragment specific (Jackson Immuno Research, Cat No. 109-095-008), was diluted 1:200 in 50 uL of FACS buffer and reacted for 1 hour on ice by blocking light. After washing 3 times with FACS buffer, resuspended in 600 uL of FACS buffer, filtered through a cell filter, and measured by FACS.

Finding the antigen to which the selected antibody binds

Immunoprecipitation was performed on Pancreatic cancer cells used for cell panning to find the antigen to which the antibody binds. After silver staining, LC-MS/MS analysis was requested from the protein core lab of Asan Medical Center (Seoul, Republic of Korea). To find the antigen to which the selected antibody binds according to the LC-MS/MS result, the genetic information was checked through literature research and whether it is a gene with high Membrane Confidence (GeneCard®: https://www.genecards.org/). In addition, human protein microarray (GENE ON BIOTECH, INC.) was requested to obtain antigen information for the selected antibody.

siRNA was used to reduce specific gene expression in cancer cells and confirmed by qPCR. Differences in antibody binding were also compared by FACS. In this experiment, cancer cells ($5X10^5$) were counted and cultured in a 60π dish the day before and transfected for 8 to 12 hours using 7uL of Lipofectamine™ 3000 Transfection Reagent (Thermofisher, Cat No. L3000015) and 5uL of siRNA (20uM). Alternatively, there are cancer cells that are transfected for 8 to 12 hours using RNAiMAX (Termofisher, Cat No. 13778030) 7.5uL and siRNA (20uM) 5uL.

After changing the media, FACS and qPCR were performed after 24-60 hours to confirm antibody binding and knockdown of specific genes. siRNA sequence information is provided in the supplement.

Quantitative real-time PCR

All RNA was extracted from cells using Tri-RNA reagent and cDNA Synthesis kit (Takara, Cat No. RR037A) was used to synthesize cDNA from RNA. The mixture of qPCR Green Mix Lo-ROX (Enzo, Cat No. ENZ-NUC103-1000), cDNA, and primers was placed in Hard-Shell PCR Plates 96-well (BIO-RAD, Cat No. HSP9601). Then, qPCR was performed in the CFX Connect Optics Module (BIO-RAD). Gene expression was normalized using the reference gene, RPL13a as an internal control. The relative expression level of the target gene was calculated using the Ct value of the target gene and the Ct value of RPL13a. Primer's information is provided in supplement table 3.

Statistics

GraphPad Prism 5.0 software was used, and statistical analysis was performed using an unpaired two-tailed Student's t-test. The data are presented as the mean ± standard deviation of triplicate measurements in a representative experiment. Values of $p<0.05$ were considered significant. Values of $p<0.05$, $p<0.005$, $p<0.0005$ are designated using *, **, and ***, respectively.

Results

Phage that is believed to specifically bind to pancreatic cancer has been selected.

The scFv library with a diversity of 1.33 x 10^{13} was prepared by cloning antibody repertoires extracted from the blood of pancreatic cancer patients with pDR-D1 as described in Materials and Methods.

To select an antibody that specifically binds only to pancreatic cancer on the cell surface, a library of 1 x 10^{12} was put and cultured using HPDE (Human Pancreatic Duct Epithelial), and library phages binding to normal pancreatic cells were previously removed through this. Library phages that were not bound to HPDE cells were retrieved and transferred to each pancreatic cancer cell for binding reaction. (Table 1) Phages were recovered from pancreatic cancer cells after panning and amplified for the next round. A total of 3 rounds were performed, and the amplification was less than expected, so a minimum of 1x10^{11} library was used in the next round.

The amount of eluted phage bound to pancreatic cancer was reduced by about 10^{-7} times compared to the injected amount, and through the amplification process, it was amplified 10^{6} times or more, so that the phage was at least 10^{11}.

The results in Table 1 show that the phages specifically bind to the pancreatic cancer cells used.

Round	구분	PancI	110621	115026
1Round	Titers of phage input(cfu*)	$1x10^{12}$	$1x10^{12}$	$1x10^{12}$
	Titers of eluted phage(cfu*)	$9x10^{4}$	$2x10^{3}$	$4x10^{4}$
	Titers after amplification(cfu*)	$1.4x10^{12}$	$4x10^{11}$	$1x10^{11}$
2Round	Titers of phage input(cfu*)	$1x10^{12}$	$4x10^{11}$	$1x10^{11}$
	Titers of eluted phage(cfu*)	$1x10^{4}$	$4x10^{4}$	$2.4x10^{6}$
	Titers after amplification(cfu*)	$7x10^{12}$	$6x10^{12}$	$2.2x10^{11}$
3Round	Titers of phage input(cfu*)	$1x10^{12}$	$1x10^{12}$	$2.2x10^{11}$
	Titers of eluted phage after infection(cfu*)	$2.4x10^{4}$	$6.2x10^{4}$	$1X10^{4}$

Table 1. Cell panning of scFv library to pancreatic cancer cells At least 10^{11} phage libraries were put in and the eluted phages were amplified for use in the next round. It was repeated three times for each cell. The elution phage bound to each pancreatic cancer cell decreased by about 10^{7} times from the injected amount. It was confirmed that phages believed to bind specifically to pancreatic cancer cells were selected.

*cfu means colony-forming unit.

Screening and grouping phagemid DNA specialized on the surface of pancreatic cancer cells.

From the second round of panning, phages eluted from each pancreatic cancer cell were cultured on an LB agar plate, and 50 to 100 colonies were randomly selected. And we collected phagemid DNA results selected in the 2nd and 3rd rounds and analyzed them to see if a total of 100 to 200 DNA sequences match each other and grouped the matching sequences (Table 2).

In the case of PANC-1, a total of 7 groups appeared, and in the PDC (patient-derived cells) 115026 and 110621, 5 groups and 18 groups appeared, respectively. However, the number of candidates found decreased further because the required scFv size was more than 700bp.[15] Referring to these details, PANC-1 used 3rd_#65 as the representative of Group 1 Ab(Antibody) of PANC-1 and 2nd_#72 as the representative of Group 2 Ab of PANC-1. These were named P-Ab65 and P-Ab72 in this paper. 115026 used 3rd_#16 as the representative of Group 1 Ab of 115026. This was named 5-Ab16 in this paper. 110621 decided to use six groups with a large population. 3rd_#05, 3rd_21, 3rd_#03, 3rd_#16, 3rd_18, and 3rd_06 were used as representatives of groups 1 to 6 Abs, respectively. These were named 6-Ab05, 6-Ab21, 6-Ab03, 6-Ab16, 6-Ab18, and 6-Ab06 in this paper. These were used for restriction enzyme cloning.

In addition, the overall results of cell panning were re-analyzed. 110621_3rd_#43 and 115026_3rd_#73 confirmed that different cells were cross-matched, and 110621_3rd_#43 was added to the cloning target. This was named 6-Ab43 in this paper.

a. PANC-1

PANC-1									
Group 1									
2nd 88	3rd 55	3rd 59	3rd 66	3rd 78	3rd 85	3rd 88	3rd 91	3rd 95	3rd 99
2nd 96	3rd 56	3rd 60	3rd 67	3rd 81	3rd 86	3rd 89	3rd 92	3rd 96	3rd 100
3rd 51	3rd 57	3rd 65	3rd 75	3rd 83	3rd 87	3rd 90	3rd 94	3rd 97	
Group 2			**Group 3**		**Group 4**	**Group 5**	**Group 6**	**Group 7**	
2nd 72	3rd 68	3rd 79	2nd 67	3rd 52	2nd 81	2nd 51	2nd 86	2nd 54	
3rd 54	3rd 69	3rd 82	2nd 78	3rd 53	3rd 70	2nd 62	2nd 90	2nd 65	
3rd 62	3rd 73	3rd 98	2nd 80	3rd 58	3rd 72	3rd 84			
3rd 63	3rd 77		2nd 94	3rd 71	3rd 80				

b. 115026

115026									
Group 1					**Group 2**		**Group 3**	**Group 4**	
3rd 16	3rd 54	3rd 81	3rd 91	3rd 98	3rd 41	3rd 82	3rd 06	3rd 64	
3rd 28	3rd 66	3rd 88	3rd 95		3rd 63		3rd 79	3rd 84	

c. 110621

110621									
Group 1						**Group 2**			
2nd 06	3rd 15	3rd 30	3rd 41	3rd 76	3rd 98	3rd 21	3rd 51	3rd 85	
3rd 05	3rd 20	3rd 31	3rd 58	3rd 87		3rd 26	3rd 59	3rd 93	
3rd 09	3rd 28	3rd 32	3rd 65	3rd 92		3rd 39	3rd 66	3rd 94	
Group 3			**Group 4**		**Group 5**	**Group 6**	**Group 7**	**Group 8**	**Group 9**
2nd 05	3rd 13	3rd 33	3rd 16	3rd 74	3rd 18	3rd 06	3rd 22	3rd 19	2nd 19
2nd 70	3rd 23	3rd 60	3rd 29	3rd 84	3rd 44	3rd 38	3rd 40	3rd 47	3rd 34
3rd 03	3rd 24		3rd 55	3rd 88	3rd 70	3rd 54	3rd 63		3rd 72
3rd 101	3rd 25		3rd 62		3rd 80	3rd 69			
Group 10	**Group 11**	**Group 12**	**Group 13**	**Group 14**	**Group 15**	**Group 16**	**Group 17**	**Group 18**	
3rd 61	3rd 49	3rd 52	3rd 77	3rd 42	3rd 57	3rd 72	2nd 45	2nd 32	
3rd 95	3rd 97	3rd 90	3rd 96	2nd 57	2nd 33	2nd 19	2nd 67	2nd 66	

Table 2. Sequencing of eluted phage DNA and grouping of cell panning results. The eluted phage from each pancreatic cancer cell was cultured on LB agar plates, and the sequence was analyzed to group matching sequences by randomly extracting DNA. This is the result of a comprehensive analysis of the eluted phages from Round 2 and Round 3. (a) PANC-1. Seven groups were found in cells of PANC-1, and only two groups had scFv size. 3rd_#65 was used as the representative in Group 1 Ab of PANC-1, and 2nd_#72 was used as the representative in Group 2 Ab of PANC-1. These were named P-Ab65 and P-Ab72 in this paper. (b) 115026. Four groups were found in cells of 115026, and only one group had scFv size. 3rd_#16 was used as the representative in Group 1 Ab of 115026. This was named 5-Ab16 (c) 110621. Eighteen groups were found in cells of 110621, and only sixteen

groups had scFv size. 3rd_#05, 3rd_#21, 3rd_#03, 3rd_#16, 3rd_#18, 3rd_#06 were used as representatives of Group 1 to 6 Ab, respectively. These were named 6-Ab05, 6-Ab21, 6-Ab03, 6-Ab16, 6-Ab18, and 6-Ab06 in this paper. (Only the graph of 110621 does not show ordinal abbreviations for round 3.) (d) Cross-matching between different cells. 110621_3rd_#43 and 115026_3rd_#73 confirmed that different cells were cross-matched, and 110621_3rd_#43 was added to the cloning target. This was named 6-Ab43 in this paper.

Cloning for scFv-Fc expression

Since pDR-Orip-Fc1 as a vector was large size, we checked instead whether the previously bonded insert size of about 700bp was cut by the restriction enzyme and whether the restriction enzyme was treated well. Then, the vector was extracted by cutting the gel with a vector size of 10 Kb or more. In the phagemid DNA, the scFv inserts appeared to be about 700bp and the gel was cut and extracted.

As a result of colony PCR after cloning, the band was shown to be about 700bp, the same size as the insert size of phagemid DNA. (Supplement figure 1)

Clones were sequenced and analyzed. The clone sequences matched the scFv sequence of phagemid DNA. (Data not shown).

Expression of scFv-Fc using HEK 293E and purification using protein G beads

HEK 293E cells were transfected using the system of mammalian expression vectors for scFv-Fc expression.

The culture supernatant was used for Coomassie staining and western blot analysis.

First, To confirm the expression of scFv-Fc, it was simply confirmed through Coomassie staining. (Supplement figure 2)

The insert size of P-Ab65 and 5-Ab16 are 700 bp or more. These bands were identified near 55kDa, which is the expression size of scFv-Fc. Therefore, candidates with an insert size of 700 bp or less in the grouping map were completely excluded from the expression target. (Supplement figure 2)

Then, Western blot was performed using goat anti-human IgG Fc antibody (HRP) to confirm the human's scFv-Fc. P-Ab65, P-Ab72, 6-Ab05, 6-Ab21, 6-Ab03, 6-Ab16, 6-Ab18, 6-Ab06 and 5-Ab161 were able to identify the band near 55kDa, expression size of scFv-Fc. (Supplement figure 2)

The supernatant cultured in serum-free using protein G beads was concentrated. (Figure 3) 5-Ab16 could not store part of the supernatant to be purified, so the supernatant used before switching to a serum-free medium as a substitute was used. So, the FBS band was confirmed. Looking at the results of the eluted solution, it was confirmed that only a single band was visible without another band, and that concentration and purification were well performed. It was also confirmed that it was eluted from the quaternary eluate, but only the tertiary eluate was collected from the primary eluate, 50% glycerol was added, stored, and the protein concentration was quantified to BCA.

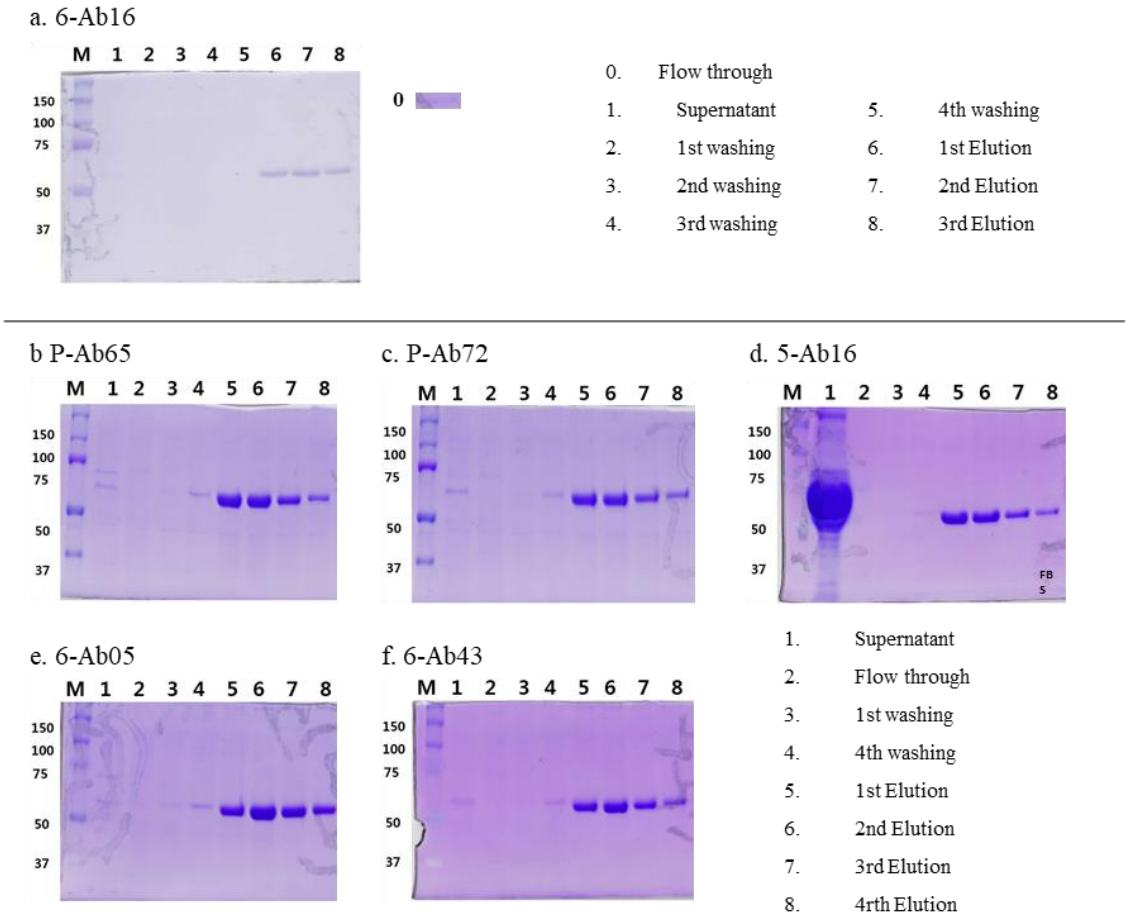


Figure 3. Confirmation after purification of scFv-Fc with protein G beads This is the result of purification using Protein G Beads with the supernatant obtained by transfection to HEK 293E. (a)6-Ab16, (b)P-Ab65, (c) P-Ab72, (d)5-Ab16 (e)6-Ab05 (f) 6-Ab43. Sample information for each well is shown next to the figure. Looking at the results of the eluted solution, it was confirmed that only a single band was visible without another band, and that concentration and purification were well performed.

Confirmation of antibody selection on the cell surface

To confirm whether the selected scFv-Fc antibody binds to Pancreatic cancer cells, the scFv-Fc culture solution and purified solution were confirmed by FACS. To confirm whether the selected scFv-Fc antibody binds to the pancreatic cancer cells, the scFv-Fc culture and purified solution were used for each target cell, and FACS was performed. (Figure 4)

In both experiments, human IgG was used as a negative control and cetuximab was used as a positive control.

First, it was confirmed that the scFv-Fc antibody was not bound to the HPDE cell used to prevent non-specific binding. (Figure 4-a) This was expressed as a dot-plot to confirm the binding rate to each cell, and it was confirmed that the results of treatment with the selected antibody were all less than 3.2% compared to 3.2% of human IgG based on the area setting.

It was confirmed that the antibodies of P-Ab65 (PANC-1 3rd_#65 Ab) and P-Ab72 (PANC-1 2nd_#72 Ab) were strongly bound in PANC-1 cells by more than 80%. (Figure 4-b) Antibodies from other cells were treated with PANC-1 cells, but the binding was not confirmed (Data not shown).

In the case of 115026 cells, 5-Ab16 (115026 3rd_#16 Ab) was not bound to 115026 cells. However, it was confirmed that the P-Ab65 and P-Ab72 antibodies were combined. (Figure 4-c)

All selected antibodies in 116021 cells did not bind to 110621 cells. However, it was confirmed that the P-Ab65 and P-Ab72 antibodies were combined. (Figure 4-d)

In addition, the selected antibodies were also tested in PDC pancreatic cancer cells (17884, 19224) and mouse pancreatic cancer cell line Pan02. As a result, binding of the P-Ab72 antibody was confirmed in 17884. (Figure 4-e) As a result, it was confirmed that P-Ab65 and P-Ab72 are combined with 19224 cells. (Figure 4-e)

Pancreatic cancer cell line derived from a mouse with Panc02 was used and it was confirmed that P-Ab72 had a bond. (Figure 4-f) Pan02 cells were not bound to cetuximab used as a positive control, so they were excluded from the results and shown in Figure.

For reference, it was confirmed whether the candidate antibody binds to other cancer cells and other normal cells.

Breast cancer cell lines MCF7 and MDA-MB-231 were tested, and PDC 050786 was also tested. As a result, more than 60% of P-Ab65 and P-Ab72 candidate antibodies were strongly bound to MCF7, MDA-MB-231 cells. In PDC 050786, P-Ab65 was bound by about 50% and P-Ab72 was weak. (Figure 5-a, Figure 5-b, Figure 5-c)

Tests on MCF10A, a normal breast cell line, confirmed binding of P-Ab65 and weak binding of P-Ab72 as well. (Figure 5-g)

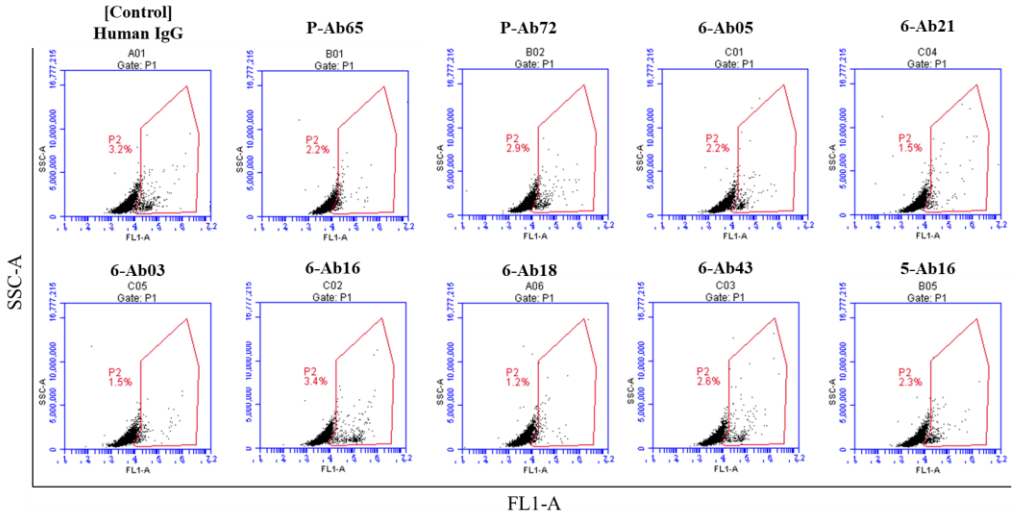
In ovarian cancer cell line SK-OV-3, P-Ab65 and P-Ab72 candidate antibodies were strongly bound at least 85%. (Figure 5-d)

In Conon cancer cell line HCT-116, P-Ab65 and P-Ab72 candidate antibodies were strongly bound at least 85%. (Figure 5-e)

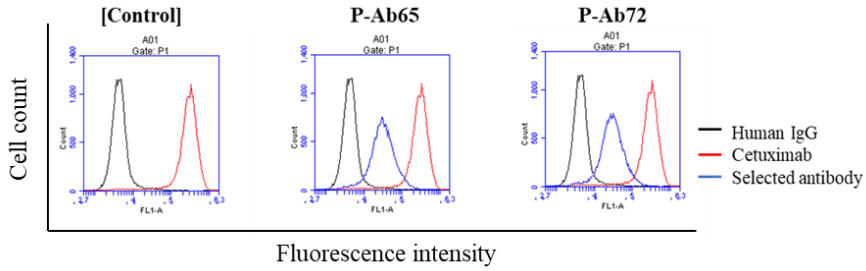
In H460, lung cancer cell line, P-Ab65 or P-Ab72 candidate antibodies were not bound. (Figure 5-f)

In HEK 239E cells used to express antibodies, P-Ab65 and P-Ab72 candidate antibodies were strongly bound at least 85%. (Figure 5-h) For reference, in the HEK 293T Lenti-X cell line tested, both P-Ab65 and P-Ab72 candidate antibodies were strongly bound at least 85%. (Figure 5-i)

a. HPDE cells



b. PANC-1 cells



c. 115026 cells

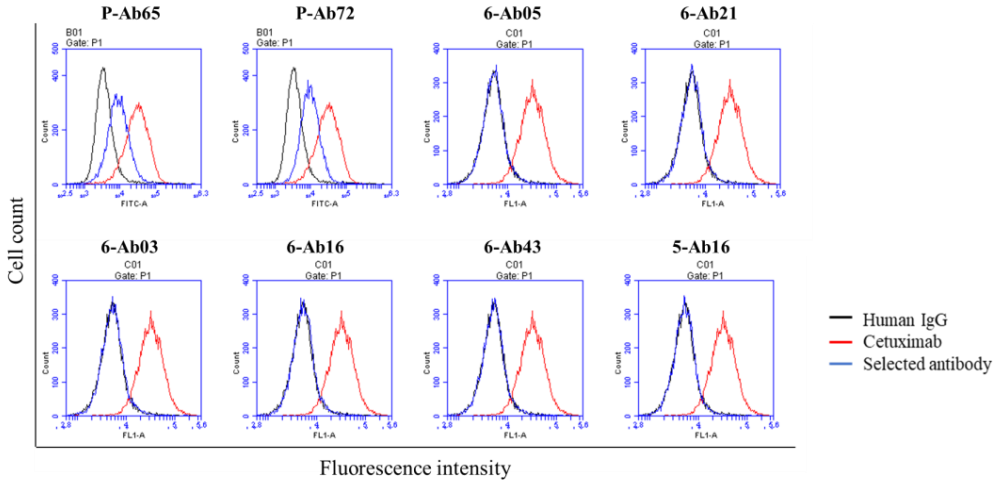
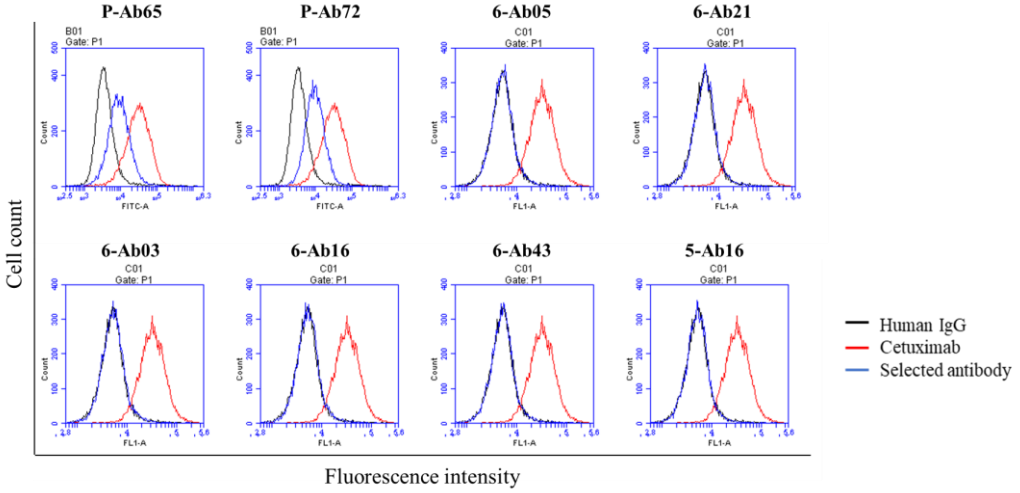
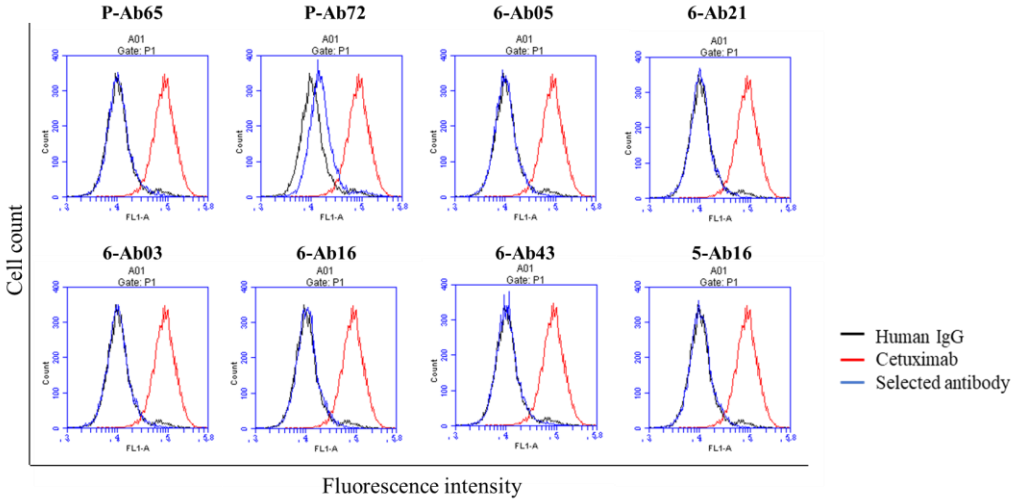


Figure 4. Confirmed the binding of candidate antibodies in pancreatic cells.

d. 110621 cells



e. 17884 cells



f. 19224 cells

g. Pan02 cells

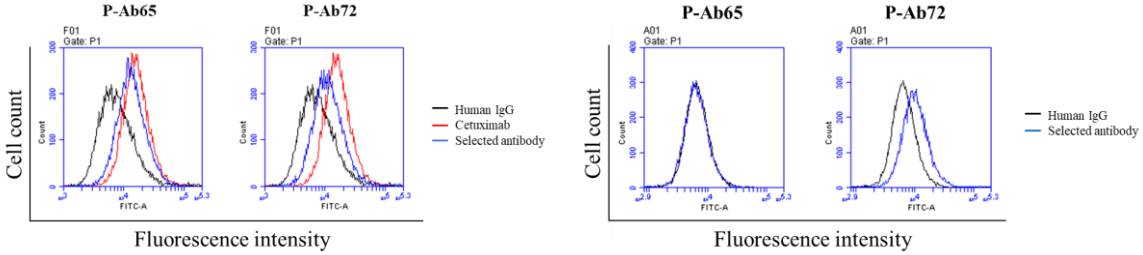


Figure 4. Confirmed the binding of candidate antibodies in pancreatic cells. FITC labels were attached to cells with selected antibodies (scFv-Fc) and analyzed using FACS. The black line is cells that combined Human IgG as a negative control. The Red line is cells that combined cetuximab as a positive control. The blue line is cells that combined supernatant, the selected antibody (scFv-Fc). (a) This is the result of binding selected antibodies to human pancreatic duct epitaxial (HPDE) cells. (b) This is the result of binding selected antibodies

to PANC-1 cells. (c) This is the result of binding selected antibodies to 115026 cells. (d) This is the result of binding selected antibodies to 110621 cells. (e) This is the result of binding selected antibodies to 17884 cells. (f) This is the result of binding selected antibodies to 19224 cells. (g) This is the result of binding selected antibodies to Panc02 cells.

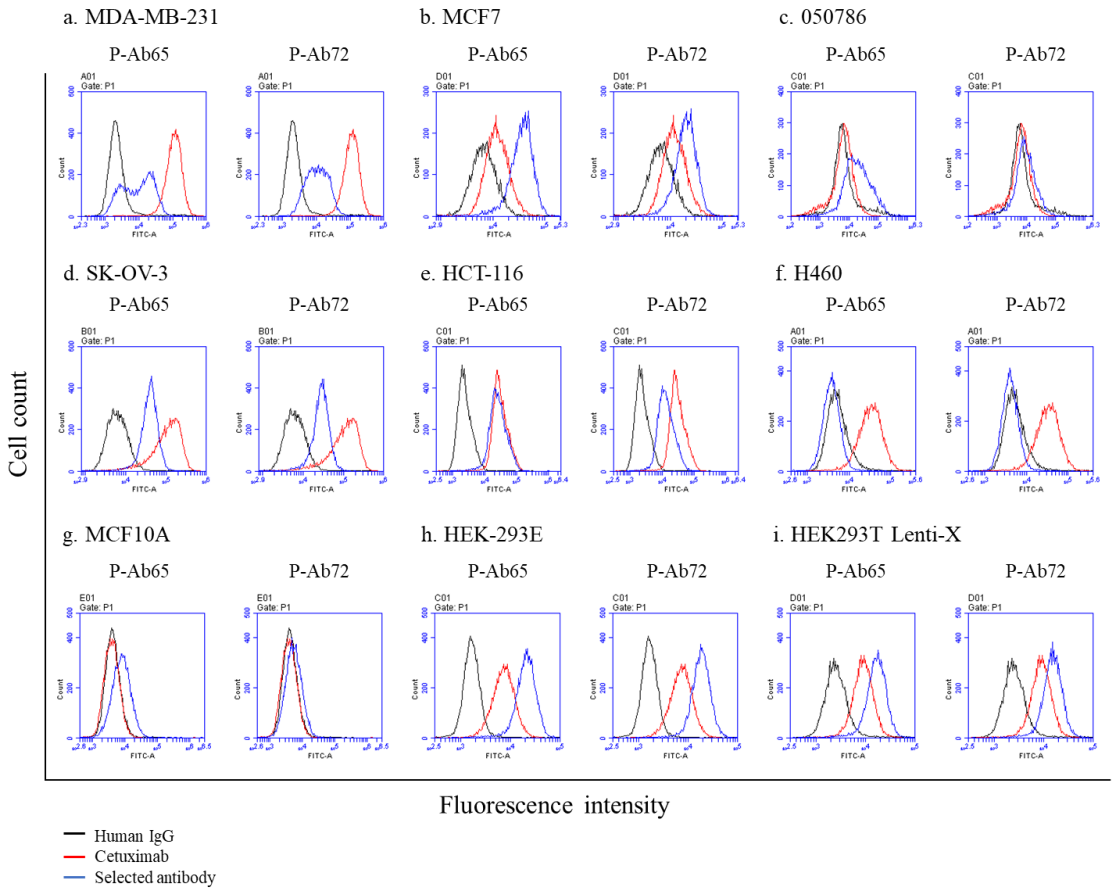


Figure 5. Confirmed the binding of selected antibodies in cells other than the pancreas FITC labels were attached to cells with selected antibodies (scFv-Fc) and analyzed using FACS. The black line is cells that combined Human IgG as a negative control. The Red line is cells that combined cetuximab as a positive control. The blue line is cells that combined supernatant, the selected antibody (scFv-Fc). **(a~c)** This is the result of binding selected antibodies to breast cancer cells (MDA-MB-231, MCF7, 050786). **(d)** This is the result of binding selected antibodies to the ovarian cancer cells, SK-OV-3. **(e)** This is the result of binding selected antibodies to the colon cancer cell, HCT-116. **(f)** This is the result of binding selected antibodies to the lung cancer cells, H460. **(g)** This is the result of binding selected antibodies to normal breast cells, MCF10A. **(h, i)** This is the result of binding the selected antibody to 293 human embryonic kidney cells, which are widely used for transformation.

Functional test according to antigen-antibody binding

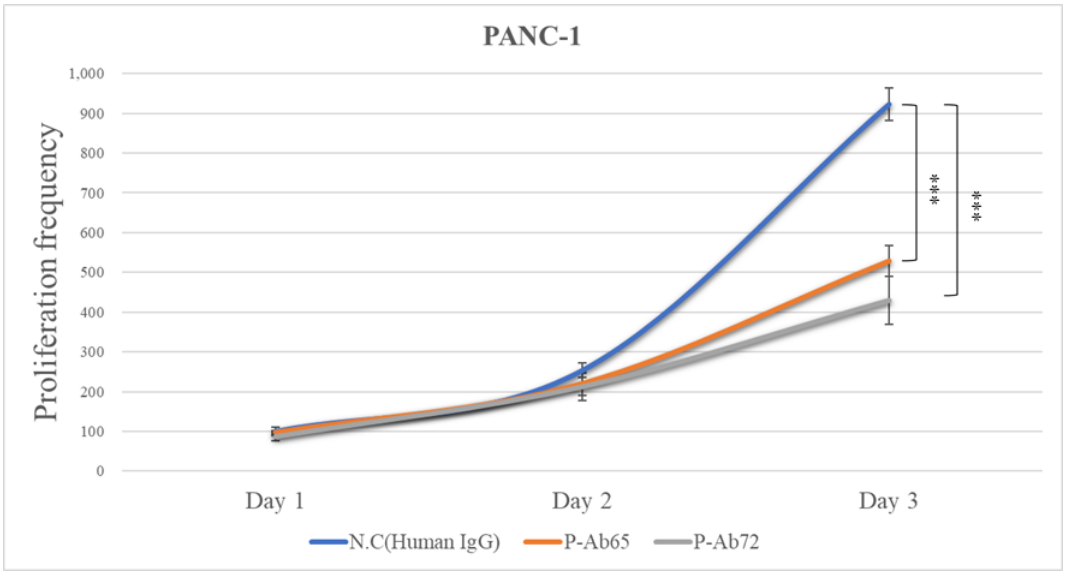
PANC-1 cells (1000 cells) were treated with 1ug of Antibody (P-Ab65 and P-Ab72) every day to confirm the change in proliferation according to the scFv-Fc antibody injection. When comparing the negative control group with the screening antibody, there was no significant impact until Day 2, depending on the presence or absence of scFv-Fc antibodies, but there was a difference in growth about twice as much as Day 3. (Figure 6-a)

Also, to confirm the result of treating the selected antibody at a low concentration (0.1, 0.2, and 0.5ug), we further confirmed the proliferation using PANC-1 and PDC (115026,19224), where the binding of the selected antibody was confirmed. In addition, the selected antibody was treated with the same concentration of antibodies to H460 cells that were not bound by the selected antibody and HPDE, a normal pancreatic cell, to confirm proliferation. Other conditions proceeded the same as the previous process. (Supplement figure 3)

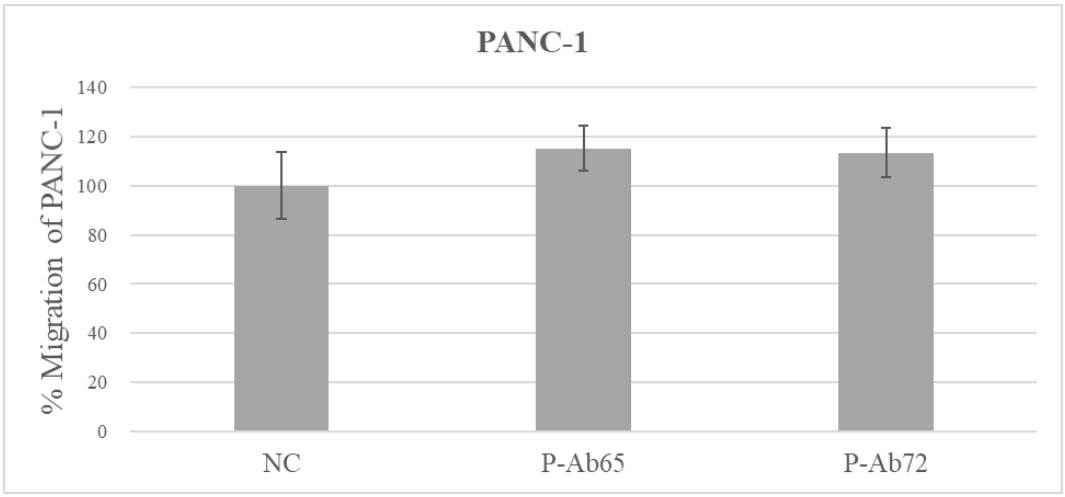
In HPDE, the proliferation of IgG-treated cells compared to the selected antibodies grew about twice as much as that of the selected antibodies. (Supplement figure 3-a) It was determined that the proliferation rate of HPDE cells was slow and difficult to use for migration confirmation, so H460 cells were tested to which the selected antibodies did not bind. In the H460 cell, there was no difference in proliferation depending on whether antibodies were added. (Supplement figure 3-b) Unlike the previous result (Figure 6), PANC-1 could not confirm the difference in proliferation due to antibodies. (Supplement figure 3-c) The results of 115026 and 19224 also could not confirm the difference in cancer cell proliferation due to antibodies. (Supplement figure 3-d, Supplement figure 3-e)

Next, to confirm the effect of migration according to the scFv-Fc antibody treatment, PANC-1 cells ($5x10^4$) were treated with P-Ab65 and P-Ab72 1ug and checked 24 hours later. As a result, it was confirmed that the migration rate was 13-15% faster in the trans-well containing the selected antibodies. (Figure 6-b) However, there was no difference in cell migration because there was no statistical significance.

a.



b.



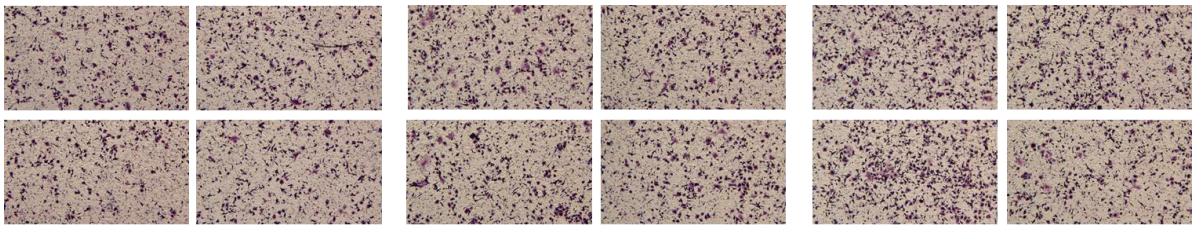


Figure 6. Changes in migration and proliferation according to antibody treatment

(a) PANC-1 proliferation assay. The graph represents the frequency of cell proliferation after 24, 48 and 72 hours.

(b) PANC-1 migration assay. The graph represents the percentage of cell migration after 24 hours.

Antigens believed to bind the selected antibody were investigated through LC-MS/MS and human protein microarrays.

Human IgG was used as a negative control, and P-Ab65 and P-Ab72 antibodies were immunoprecipitated into PANC1-1 cells, respectively. And 6-Ab05 and 6-Ab16 antibodies were immunoprecipitated into 110621 cells, respectively. This was silver-stained after electrophoresis on 10% SDS PAGE. (Supplement figure 4) Then, LC-MS/MS analysis was performed at Protein Core Lab. (Figure 7, Supplement table 4)

Except for 451 genes bound to IgG in PANC-1, 48 genes unique to P-Ab65, 22 genes unique to P-Ab72, and 12 genes common to P-Ab65 and P-Ab72 were confirmed.

In 110621, excluding 522 genes bound to IgG, there were 136 genes unique to 6-Ab05, 114 genes unique to 6-Ab16, and 123 genes common to 6-Ab05 and 6-Ab16 was confirmed. However, it was concluded that the antibodies panned at 110621 were not bound to 110621 cells, so no further tests were conducted.

To find the antigen to which the selected antibody binds according to the LC-MS/MS result, the genetic information was checked through literature search and whether the gene had high membrane confidence was confirmed. Then the siRNA of the gene most distributed in the membrane was investigated and designed. (Supplement table 2) [16~33]

Human protein microarray (GENE ON BIOTECH, INC) was requested to find the antigen to which the antibody binds by another method. (Supplement figure 6)

This confirmed the A-Score based on the signal intensity indicated by about 21,000 protein spots. the top 1% proteins were selected in the A-Score and statistically significant binding was confirmed through the S-score. A-score (affinity score) means the normalized signal intensity of dual spots. S-score (specificity score) means the difference between the A-scores of selected antibodies (P-Ab65 and P-Ab72) the protein ranked next to it.

As a result, it was confirmed that a total of 20 proteins were bound to the P-Ab65 antibody, and a total of 49 proteins were bound to the P-Ab72 antibody.

A comprehensive analysis of human protein microarray and LC-MS/MS results found common genes to which antibodies bind. (Supplement table 5)

a. PNAC-1 b. 110621

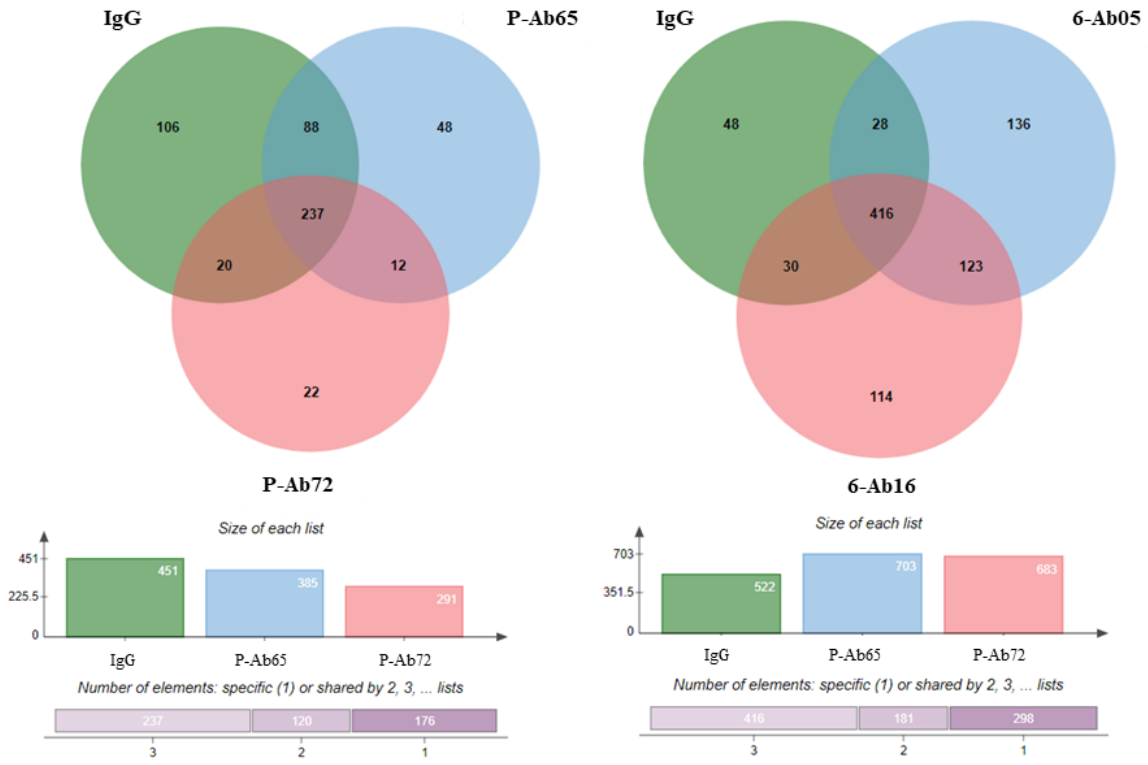


Figure 7. Analysis of LC-MS/MS results to find the antigen to which the selected antibody binds.

(**a) PANC-1.** Except for 451 genes bound to IgG in PANC-1, 48 genes unique to P-Ab65 (=Ab 1), 22 genes unique to P-Ab72 (=Ab 2), and 12 genes common to P-Ab65 and P-Ab72 were confirmed. **(b) 110621.** In 110621, excluding 522 genes bound to IgG, there were 136 genes unique to 6-Ab05 (=Ab 1), 114 genes unique to 6-Ab16 (=Ab 2), and 123 genes common to 6-Ab05 and 6-Ab16 were confirmed.

Knockdown the gene of the antigen believed to bind the selected antibodies and confirm it through FACS.

Small interfering RNA (siRNA) was transfected and RFP was co-transfected to confirm the transfection efficiency. The RFP luminous rate was confirmed to be more than 50% through a fluorescence microscope. As another method, the mRNA expression level of the siRNA target gene was analyzed through qPCR. (Supplement figure 7-b, Figure 8-b, Supplement table 3, Supplement figure 5) The difference in the binding force of the selected antibody was analyzed by FACS. (Figure 8-a)

As a result of using the Lipofectamine ™ 3000 Transfection Reagent, it was confirmed that antibodies bound to siRNA were more bound to cells compared to untreated cells and could not be used in binding reduction confirmation experiments. Therefore, the produced siRNA was used only to determine whether knockdown was good. (Supplement figure 7)

When RNAiMAX reagents were used, the values of binding the selected antibody to the untreated cells and the cells treated with siRNA NC (negative control) were consistent.

As a result of checking using RNAiMAX reagents to find antigens, antibody-antigen binding was reduced in cells that knockdown the CALML5, DSP, GJA5, GRP1, LARP7, MUC5AC, RDX and SERPINA4 genes in PANC-1 cells.

Several genes whose binding was confirmed in the stomach were reconfirmed in other cells.

As a result of the knockdown of genes in HEK 293T Lenti-X cells, antibody-antigen binding was reduced in cells treated with DSP, LARP7, and MUC5AC siRNA. (GJA5 siRNA with reduced binding in PANC-1 was not tested.)

As a result of the knockdown of genes in HEK 293E cells, antibody-antigen binding was reduced in cells treated with GRPEL1, MUC5AC, SERPINA4 siRNA. (DSP, GJA5, LARP7, RDX siRNA was not tested.)

As a result of the knockdown of genes in PDC 115026 cells, antibody-antigen binding was reduced in cells treated with DSP, GRPEL1, LARP7, MUC5AC, SERPINA4 siRNA. (CALML5, GJA5 siRNA was not tested.)

As a result of the knockdown of genes in PDC 19224 cells, antibody-antigen binding was reduced in cells treated with GRPEL1, LARP7, MUC5AC, SERPINA4 siRNA. (CALML5, DSP, GJA5 siRNA was not tested.)

Summarizing the results, the most likely genes for antigens are MUC5AC, LARP7, DSP, SERPINA4, and GRPEL1, where differences in antigen-antibody binding have been identified several times.

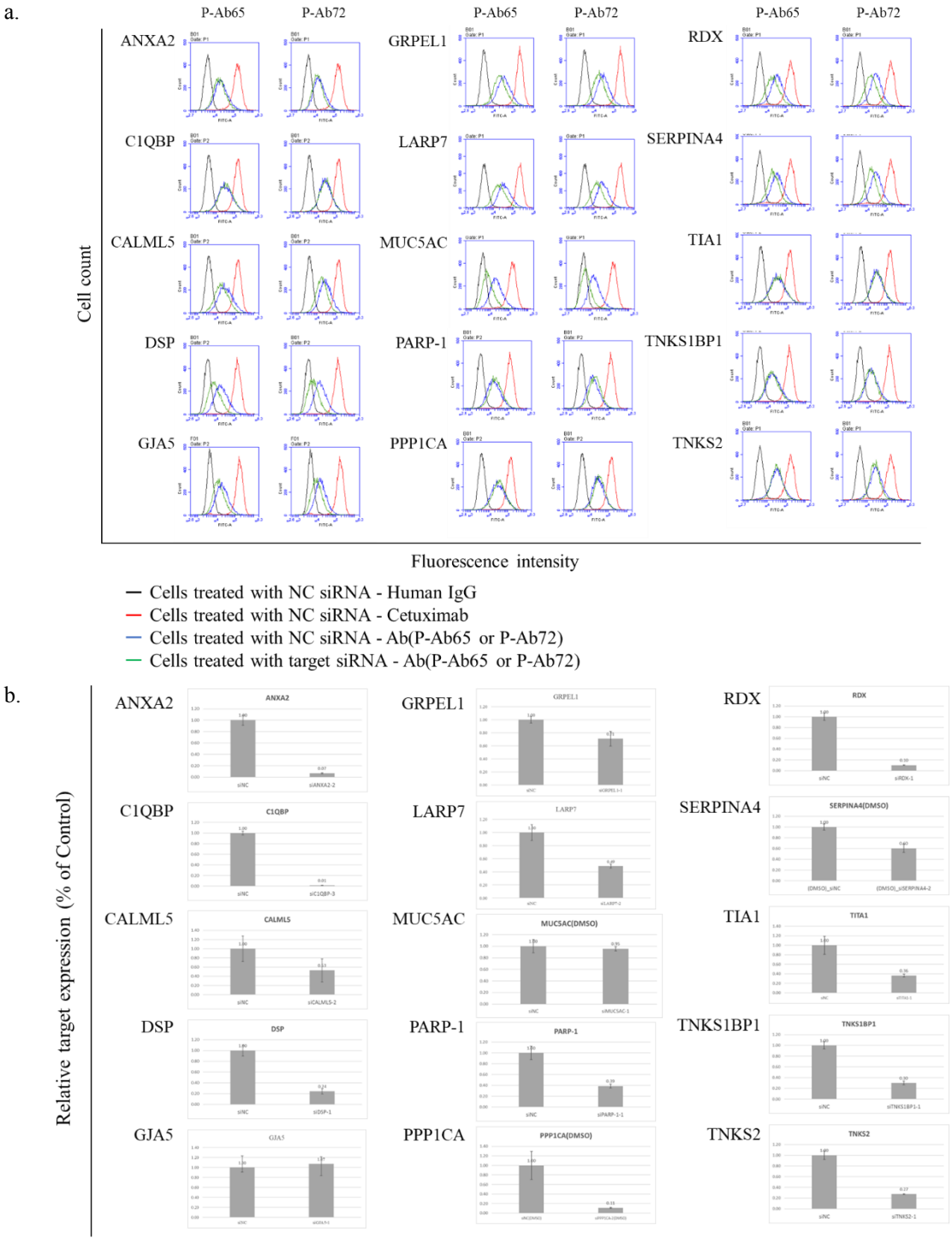


Figure 8. Confirmed binding affinity and knockdown after siRNA treatment in PANC-1 cells. Knockdown was performed using siRNA to confirm binding reduction to identify the gene of the antigen that the selected antibody is expected to bind to. It was performed to find antigens in PANC-1 cells, cells that screened antibodies through cell panning. **(a)** FACS was used to check the reduction in binding. FITC labels were attached to cells

with selected antibodies (scFv-Fc) and analyzed using FACS. The black line is negative controls that reacted with human IgG to cells treated with siRNA NC. The Red line is positive control that reacted with cetuximab to cells treated with siRNA NC. The blue line is the control group that reacted with selected antibody (scFv-Fc) to cells treated with siRNA NC. The green line is the experiment group that reacted with selected antibody (scFv-Fc) to cells treated with target siRNA. **(b)** It shows the value of knockdown by siRNA using quantitative real-time PCR. It was analyzed based on siRNA NC.

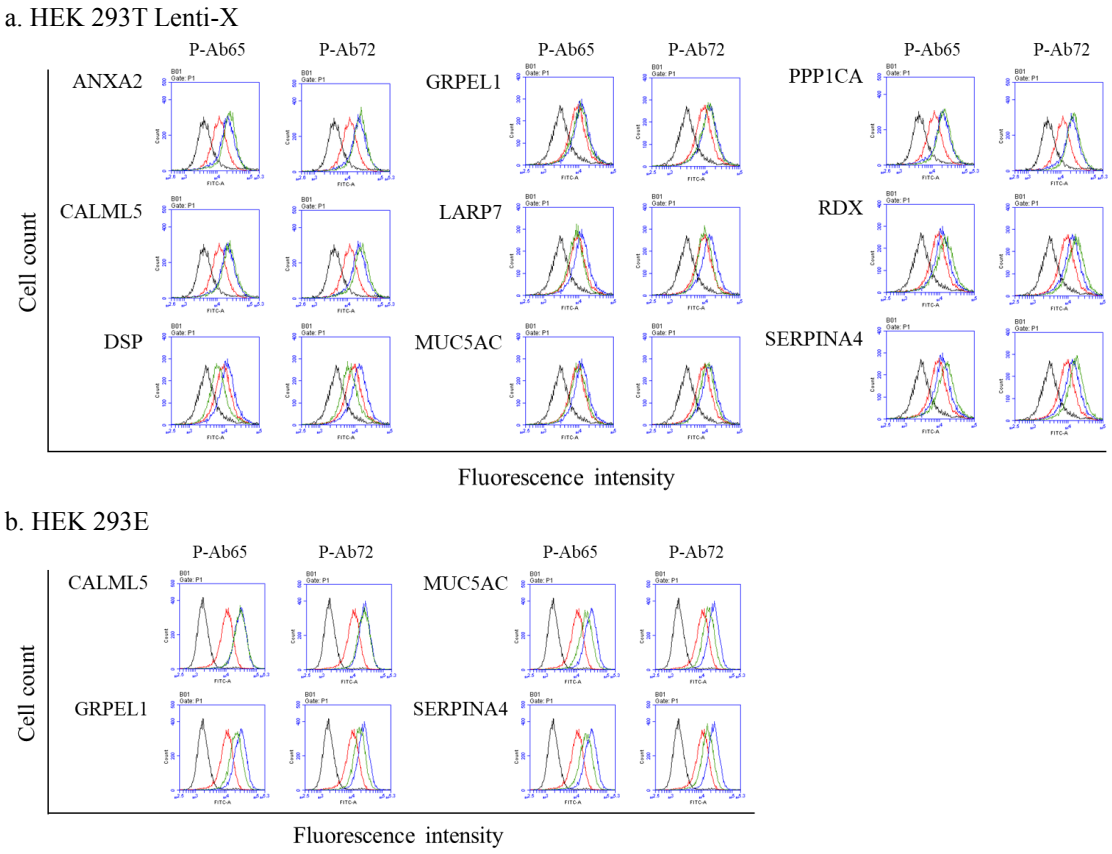


Figure 9. Knockdown specific genes in cells other than PANC-1 cells to determine antigen-antibody binding differences.

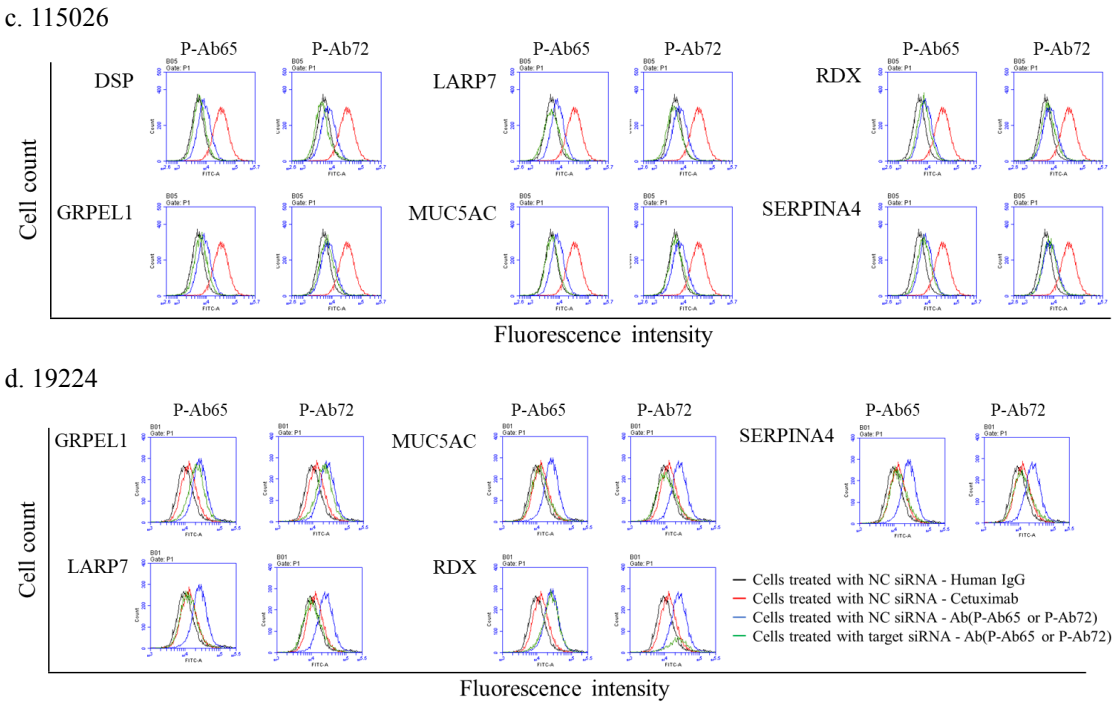


Figure 9. Knockdown specific genes in cells other than PANC-1 cells to determine antigen-antibody binding differences. Knockdown was performed using siRNA to confirm binding reduction to identify the gene of the antigen that the selected antibody is expected to bind to. Several siRNAs with reduced antigen-antibody binding in PANC-1 cells were repeatedly tested in other cells. FACS was used to check the reduction in binding. FITC labels were attached to cells with selected antibodies (scFv-Fc) and analyzed using FACS. The black line is negative controls that reacted with human IgG to cells treated with siRNA NC. The Red line is positive control that reacted with cetuximab to cells treated with siRNA NC. The blue line is the control group that reacted with selected antibody (scFv-Fc) to cells treated with siRNA NC. The green line is the experiment group that reacted with selected antibody (scFv-Fc) to cells treated with target siRNA. **(a) HEK 293T Lenti-X cells with siRNA. (b) HEK 293E cells with siRNA. (c) PDC 115026 cells with siRNA. (d) PDC 19224 cells with siRNA.**

Discussion

Using the scFv library made from the blood of 10 pancreatic cancer patients, we found 29 groups that were non-specific to normal pancreatic cells (HPDE) and that specifically bind to each pancreatic cancer cell through phage display technology. Of these, only 19 groups had a sequence size of scFv (greater than 700 bp). We selected 2 groups from PANC-1, 1 group from 115026, and 6 groups from 110621, and also included one additional clone present in crossover cells.

The selected scFv (antibody fragment) had a difference in base size within 25bp, and there was a difference in protein expression size of antibodies screened in PANC-1 and antibodies screened in other cells. The antibodies selected in 110621 and 115026 were confirmed to have no interruption in translation into proteins, but the binding was not confirmed in FACS, which is presumed to be due to the characteristics of PDC cells. Perhaps it can be assumed that the cells used for cell panning and the cells used for FACS are not bound because they are not in the same state. As indicated by FACS results, more than 70% of strong bonds were identified in P-Ab65 and P-Ab72 antibodies to PANC-1 cell, and binding was also identified in PDC (115026, 110621, 17884, 19224) cell. It also suggests that antibodies that bind to PDC can bind to each pancreatic cancer patient. (Figure 4) PDC (Patient-Derived Cell) is a cancer cell isolated from a specific patient and is more similar to the in vivo environment than cancer cell lines. When the selected antibody was tested on these cells, efficacy and effects could be predicted by reflecting the effects of the environment occurring in the body.

In addition, the P-Ab72 antibody was bound to Pan02, a mouse-derived Pancreatic canceler cell. This means that it can be used to verify the effectiveness of immunotherapy by creating an allograft model and conjugating CAR-T etc. to the selected antibody in the future.

The selected antibody was also found to bind to other cancers (breast cancer, ovarian cancer, colon cancer), and no binding was confirmed in lung cancer cells. In MCF10A, a normal breast cell, weak binding was confirmed only in the P-Ab65 antibody. In summary, P-Ab72 antibodies could also be used as markers for early cancer diagnosis by confirming binding in all PDC cells and cancer cell lines except lung cancer, but they are strongly bound in human embryonic kidney cells HEK-293E and HEK 293T Lenti-X, which would likely be used only in specific tissues rather than in drug-ant conjugate development. (Figure 5)

When treating the antibody selected in PANC-1, it was confirmed that the proliferation decreased by 50% compared to IgG, but when the concentration of the treated antibody was lowered, there was no significant difference in proliferation. However, in HPDE cells, which are normal pancreatic cells in humans, proliferation decreased by 50% compared to IgG. It is necessary to reconfirm whether the selected antibody inhibits the proliferation of normal cells, loses due to daily antibody replacement, and its cell characteristics. In FACS, the binding of selected antibodies was not confirmed in HPDE and H460 cells. Therefore, I thought that the proliferation change should have been similar if the two cells had no other effect than the selected antibody. (Supplement figure 3)

The migration test results were not significant. In addition, it is necessary to check the difference in migration by simultaneously culturing PNAC-1 cells and PaCSC (pancreatic cancer stem cells). PaCSCs plays a fundamental role in the initiation and development of PDAC (pancreatic ductal adenocarcinoma), and these cells are largely responsible for the aggressive, chemoresistant and metastatic nature of this cancer. [41] Therefore, an additional experiment is needed to confirm the difference when treating the selected antibody co-cultured with PaCSC.

A band to which antibodies bind specifically was identified through IP, and it was visually confirmed that there was an antigen to which the selected antibody binds. (Supplement figure 4) We analyzed this by LC/MS-MS and selected a method of finding antigens by knocking out genes that are widely distributed in the membrane.

siRNA was produced by selecting the target gene, and the difference in binding strength was confirmed by transfecting the siRNA into cells. Among them, in several experiments, there was a difference in binding strength in cells in which the TNKS1BP1 gene was knocked down. Here, we assumed that the assayed antibody does not bind directly, but rather through a substrate. Despite the knockdown of TNKS1BP1-related genes (PARP-1, TNKS2), no differences in binding affinity were identified. [34, 35] The problem was that TNKS1BP1 was also repeated several times, but unlike before, there was no difference in binding strength due to knockdown.

We compared cells treated with siRNA negative control and non-treated cells and recognized that this affects the binding strength of the selected antibody. This is a problem with finding binding antigens using FACS, so we solved it by changing the reagent used for transfection.

To narrow the range of antigens (genes) identified in the LC-MS/MS results, additional human protein microarray analysis was conducted, and the antigens were investigated intensively by investigating common genes from the two experiments. (Supplement table 5)

The difference in antigen-antibody binding according to gene knockdown was confirmed several times, and the antigen candidate of the selected antibody was determined through this. The summary of the results is listed in Supplement Table 6. The most likely genes for antigens are MUC5AC, LARP7, DSP, SERPINA4, and GRPEL1. Among them, GRPEL1 and MUC5AC did not confirm a decrease in expression in qPCR results, so the target expression will be confirmed using other primers or through Western blots.

In particular, MUC5AC, which identified the most antigen-antibody binding differences, is secretory mucin that is abnormally expressed in various cancers, and differential expression of MUC5AC improves tumor formation. [43, 44] In pancreatic cancer cells, high MUC5AC expression is associated with increased migratory potential. [45] MUC5AC is an important determinant of poor prognosis, especially in patients with KRAS mutant tumors. [42]

Western blot will be performed by attaching a protein thought to be an antigen to the sample used for IP.

If the five candidate antigens are not antigens of the selected antibody, magnetic activated cell sorting (MACS) will be performed on human emblem kidney cells with a binding rate of 90% or more to find the antigen. (Figure 5)

Once the antigen has been identified, A patient-derived xenograft (PDX) model will be prepared by injecting pancreatic cancer cells into the mouse pancreas, and the effect on cancer treatment will be confirmed by administering the selected antibody or simultaneously administering the selected antibody and immunotherapeutic agent.

Supplementary table

Supplement table 1. Primer information used from library construction to cloning confirmation

#	Purpose	Primer name	Sequence
1	Confirmation of phagemid DNA sequence	pDR-D1_F	GCAATTAATGTGAGTTAGCTCACT
		pDR-D1_R	CCCTTATTAGCGTTTGCCATCTT
2	Confirmation of cloning (Common)	pDR-OriP-Fc1_F	TGGAGACAGACACAATCCTGC
		pDR-OriP-Fc1_R	TCCTTGCCATTCAGCCAGTC
	Confirmation of cloning (Special production for a group of 110621,115026)	3,43,54_F	AAGCAGCTCCAACATCGGAA
		6,54_F	CCTGAGATCTGACGACACGG
		16_F	CACCCTCTGGATTCGCCTTT

Supplement table 2. siRNA information

#	Target gene	siRNA name	siRNA sequence
1	Genolution negative control	siNC_1	CCUCGUGCCGUUCCAUCAGGUAGUU
2	ANXA2	siANXA2_1	UGAGGGUGACGUUAGCAUUACUU
		siANXA2_2	CGGGAUGCUUUGAACAUUGAAUU
3	C1QBP	siC1QBP_1	GGAGGGAUACAAACUAUACACUCAAUU
		siC1QBP_2	GAAGGCCCUUGUGUUGGACUGUCAUUU
		siC1QBP_3	ACUGGCGAGUCUGAAUGGAAGGAUAUU
4	CALML5	siCALML5_1	AAGGCUUUCUCCGCGGUUGACUU
		siCALML5_2	GGCCCAGCUAAGGAAACUCAUUU
5	DSP	siDSP_1	ACCGUCACUGAGCUAGUAGAUUCUGUU
		siDSP_2	AAGUUUGCUAUUCUGGCAAUAAACUUU
6	GJA5	siGJA5-1	AAGCCUUUCAGAUUACUCAUGUU
		siGJA5-2	AAGGUCUGGCUCACUGUCCUCUU
7	GRPEL1	siGRPEL1_1	AAGGACUUGUUGGAGGUGGCAUU
		siGRPEL1_2	GCUGAAUGAUUGGCGUCUGUUUU
8	LARP7	siLARP7_1	NNGAAGAAAGGCCGAAUGAAAUU
		siLARP7_2	AAGCAACACCAGCAUCAGUAAUU
9	MUC5AC	siMUC5AC_1	UUUGAGAGACGAAGGAUACUU
		siMUC5AC_2	GAAAUCCAGGACAACCACUUUUU
10	PARP-1	siPARP-1_1	GCAGCUUCAUAACCGAAGAUUUUUU
11	PPP1CA	siPPP1CA_1	CAAGAUCUGCGGUGACAUAUUUU
		siPPP1CA_2	AAGCACGACUUGGACCUCAUCUU
12	RDX	siRDX_1	UAGUUUGUGUUGUUCCAAUACACGCUU
13	SERPINA4	siSERPINA4_1	AAGGCCACCUUGGACGUGGAUUU
		siSERPINA4_2	GCUAAUGAGGUGGAACAACUUUU
14	TIA1	siTIA1_1	AAGAGUUGCAGAAUUAGAGCUUCUGUU
		siTIA1_2	AACAACUAAUGCGUCAGACUUUUUU
15	TNKS1BP1	siTNKS1BP1_1	UAUCCAAGCGCUCUUCCCAAACUCCUU
		siTNKS1BP1_2	AAGACGAGGAGUAAUCUUCACCCUGUU
16	TNKS2	siTNKS2_1	GAGGGUAUCUCAUUAGGUAUUUU
		siTNKS2_2	AGCUCAUAAUGAUGUUGUUGAAGUAUU

Supplement table 3. PCR primer sequences for knockdown confirmation using siRNA

#	Target	Primer name	Sequence
1	ANXA2	ANXA2_F	GAGCGGGATGCTTTGAACATT
		ANXA2_R	TAGGCGAAGGCAATATCCTGT
2	C1QBP	C1QBP_F	AGTGCGGAAAGTTGCCGGGGA
		C1QBP_R	GAGCTCCACCAGCTCATCTGC
3	CALML5	CALML5_F	CCTTCGACCAGGATGGCGAC
		CALML5_R	TCCCATCCACCGACCAGGTT
4	DSP	DSP_F	ACAGTGAAATATCTGGCAAACGAG
		DSP_R	CTCATAAGTCAGTCGGGTGAT
5	GJA5	GJA5-F	GATGGCCTTGGGTTGCCCTT
		GJA5-R	AGGAGCTGCAGGGTGACAGA
		GJA5-F2	TTTCAAACAGTCCCTCCTGGG
		GJA5-R2	GAAAGCCTGGTCGTAGCAGA
6	GRPEL1	GRPEL1_F	AGGCCTTGTTCCACACACCG
		GRPEL1_R	TGGGCAACCGGCTTTTCTGT
7	LARP7	LARP7_F	TTTGCGTTTGTGGAATTTGA
		LARP7_R	AGGCTGGAATGGGCTTATTT
8	MUC5AC	MUC5AC_F	CTACTTCTGCGGTGCGGAGG
		MUC5AC_R	ACTGGCCCTCGGTGTTGTTG
9	PARP-1	PARP-1_F	AAGGCGAATGCCAGCGTTAC
		PARP-1_R	GGCACTCTTGGAGACCATGTCA
10	PPP1CA	PPP1CA_F	CAGGGTCCTGACACCCCATT
		PPP1CA_R	AGGTAAAAGAGACGCCACGG
11	RDX	RDX_F	TGCACCTCG TCTGAGAATCA
		RDX_R	CTCTAATTGTGCCCTTTCCAAC
12	SERPINA4	SERPINA4_F	TGGAGACAGGTGAGGGCTCC
		SERPINA4_R	CCCTCAAGGATCTGGCTGCG
		SERPINA4_F2	ACAGACTGTGCCGCCTGAG
		SERPINA4_R2	AAAAACACGGTTGCGTCTCC
13	TIA1	TIA1_F	CCATGGATGGGACCAAATTA
		TIA1_R	TTTCATACCCTGCCACTCGAT
14	TNKS1BP1	TNKS1BP1_F	CTGCTCTGAGGGACTCCTTG
		TNKS1BP1_R	CTGGGTCTCCTCTAGGGCTT
15	TNKS2	TNKS2_F	ATCTGCTCTGCCCTCTTGTTACAA
		TNKS2_R	GCTAAAATCTACTCCTGGAACCTC

Supplement table 4. Summary of LC-MS/MS analysis results by binding P-Ab65 and P-Ab72 antibodies to PANC-1 cells.

Elements only in _ P-Ab65 antibody :	
28S ribosomal protein S22, mitochondrial MRPS22	Histone H2A type 1 H2AC11
28S ribosomal protein S31, mitochondrial MRPS31	Immunoglobulin lambda variable 1-47 IGLV1-47
3'-5' RNA helicase YTHDC2 YTHDC2	Isoform 2 of Cysteine-rich protein 2 CRIP2
39S ribosomal protein L11, mitochondrial MRPL11	Isoform 2 of Serine/threonine-protein phosphatase PP1-alpha catalytic subunit PPP1CA
39S ribosomal protein L17, mitochondrial MRPL17	Isoform 3 of La-related protein 7 LARP7
40S ribosomal protein S30 FAU	Isoform 4 of Acyl-coenzyme A thioesterase 9, mitochondrial ACOT9
60S ribosomal protein L22-like 1 RPL22L1	Isoform 4 of CUGBP Elav-like family member 1 CELF1
Aldehyde dehydrogenase family 3 member B1 ALDH3B1	Isoform 5 of Nuclear factor 1 B-type NFIB
Bleomycin hydrolase BLMH	Kallistatin SERPINA4
Bystin BYSL	La-related protein 4B LARP4B
Calmodulin-like protein 5 CALML5	Lupus La protein SSB
C-Maf-inducing protein CMIP	Nucleolar RNA helicase 2 DDX21
Complement component 1 Q subcomponent-binding protein, mitochondrial C1QBP	PDZ domain-containing protein GIPC1 GIPC1
Core histone macro-H2A.1 MACROH2A1	PHD finger protein 6 PHF6
Desmoplakin DSP	Poly [ADP-ribose] polymerase 1 PARP1
Dynein heavy chain 10, axonemal DNAH10	Protein LSM14 homolog B LSM14B
Eukaryotic initiation factor 4A-III EIF4A3	Protein SETSIP SETSIP
Eukaryotic translation initiation factor 2 subunit 2 EIF2S2	RuvB-like 2 RUVBL2
Eukaryotic translation initiation factor 3 subunit E EIF3E	Splicing factor U2AF 65 kDa subunit U2AF2
Eukaryotic translation initiation factor 3 subunit F EIF3F	Squamous cell carcinoma antigen recognized by T-cells 3 SART3
Fragile X mental retardation syndrome-related protein 1 FXR1	Staphylococcal nuclease domain-containing protein 1 SND1
Gap junction alpha-5 protein GJA5	Thioredoxin-dependent peroxide reductase, mitochondrial PRDX3
GrpE protein homolog 1, mitochondrial GRPEL1	Trifunctional enzyme subunit alpha, mitochondrial HADHA
Heterogeneous nuclear ribonucleoprotein U-like protein 2 HNRNPUL2	Trinucleotide repeat-containing gene 6B protein TNRC6B
Elements only in _ P-Ab72 antibody :	
Immunoglobulin heavy constant gamma 3 IGHG3	Isoform 3 of Bcl-2-like protein 2 BCL2L2
Immunoglobulin heavy constant gamma 4 IGHG4	Immunoglobulin kappa variable 3D-7 IGKV3D-7
Keratin, type II cytoskeletal 73 KRT73	Ubiquitin-like modifier-activating enzyme 1 UBA1
Immunoglobulin lambda constant 7 IGLC7	182 kDa tankyrase-1-binding protein TNKS1BP1
Mucin-5AC MUC5AC	AP-2 complex subunit alpha-1 AP2A1
Isoform 2 of Annexin A2 ANXA2	Immunoglobulin kappa variable 3D-11 IGKV3D-11
Immunoglobulin lambda variable 3-21 IGLV3-21	Eukaryotic translation initiation factor 5B EIF5B
Putative 40S ribosomal protein S10-like RPS10P5	Ataxin-2 ATXN2
Immunoglobulin kappa variable 3D-20 IGKV3D-20	ZAR1-like protein ZAR1L
Elongation factor 1-alpha 2 EEF1A2	14-3-3 protein epsilon YWHAE
28S ribosomal protein S25, mitochondrial MRPS25	39S ribosomal protein L3, mitochondrial MRPL3
Common elements in _ P-Ab65, P-Ab72 antibody:	
5'-3' exoribonuclease 2 XRN2	Isoform Short of Nucleolysin TIA-1 isoform p40 TIA1
Arachidonate 12-lipoxygenase, 12R-type ALOX12B	Keratin, type I cytoskeletal 16 KRT16
ATPase family AAA domain-containing protein 3A ATAD3A	Muscleblind-like protein 1 MBNL1
Heat shock 70 kDa protein 6 HSPA6	RNA-binding motif, single-stranded-interacting protein 2 RBMS2
Isoform 3 of Protein SEC13 homolog SEC13	TATA-binding protein-associated factor 2N TAF15
Isoform 5 of Radixin RDX	Trifunctional enzyme subunit beta, mitochondrial HADHB

Supplement table 5. Common genes of human protein microarrays and LC-MS/MS to find antigens to which antibodies bind.

Panc1 group 1			Panc1 group 2		
#	A-Score ranking	Gene	#	A-Score ranking	Gene
1	118	FXR1	1	50	BLMH
2	171	MRPL11	2	80	ATAD3A
3	323	ALDH3B1	3	185	RBMS2
4	425	ZAR1L	4	287	BYSL
5	503	BYSL	5	312	MRPL11
6	514	RBMS2	6	513	SEC13
7	732	ATAD3A	7	678	NFIB
8	795	NFIB	8	690	FXR1
9	1697	GIPC1	9	990	ANXA2
10	2165	HADHB	10	1062	MRPS25
11	2742	U2AF2	11	1257	MRPS22
12	3289	PRDX3	12	1413	HADHB
13	3433	UBA1	13	1428	TAF15
14	3668	ANXA2	14	1884	U2AF2
15	4012	EIF3F	15	1922	EIF3F
16	4613	MRPS31	16	2354	GIPC1
17	4909	TAF15	17	2368	UBA1
18	4940	TIA1	18	2554	RPL22L1
19	5092	HADHA	19	2565	MRPL3
20	5313	IGHG3	20	2574	TIA1
21	6004	GJA5	21	2705	ZAR1L
22	6226	EIF2S2	22	2718	PRDX3
23	6240	LARP4B	23	3047	EIF4A3
24	6424	MRPL17	24	3097	LARP7
25	6431	DDX21	25	3500	SND1
26	7621	AP2A1	26	3615	MBNL1
27	7993	BCL2L2	27	3837	ALDH3B1
28	8113	HNRNPUL2	28	4142	EIF3E
29	8494	SSB	29	5125	BCL2L2
30	9744	MRPL3	30	5557	PHF6
31	9779	MBNL1	31	5603	PARP1
32	10619	C1QBP	32	6141	EEF1A2
33	10835	MRPS25	33	6347	GJA5
34	10937	CRIP2	34	7619	PPP1CA
35	10946	YWHAE	35	7670	EIF2S2
36	11289	RDX	36	8336	SSB
37	11724	EIF3E	37	8975	LARP4B
			38	10056	HADHA
			39	10368	HNRNPUL2

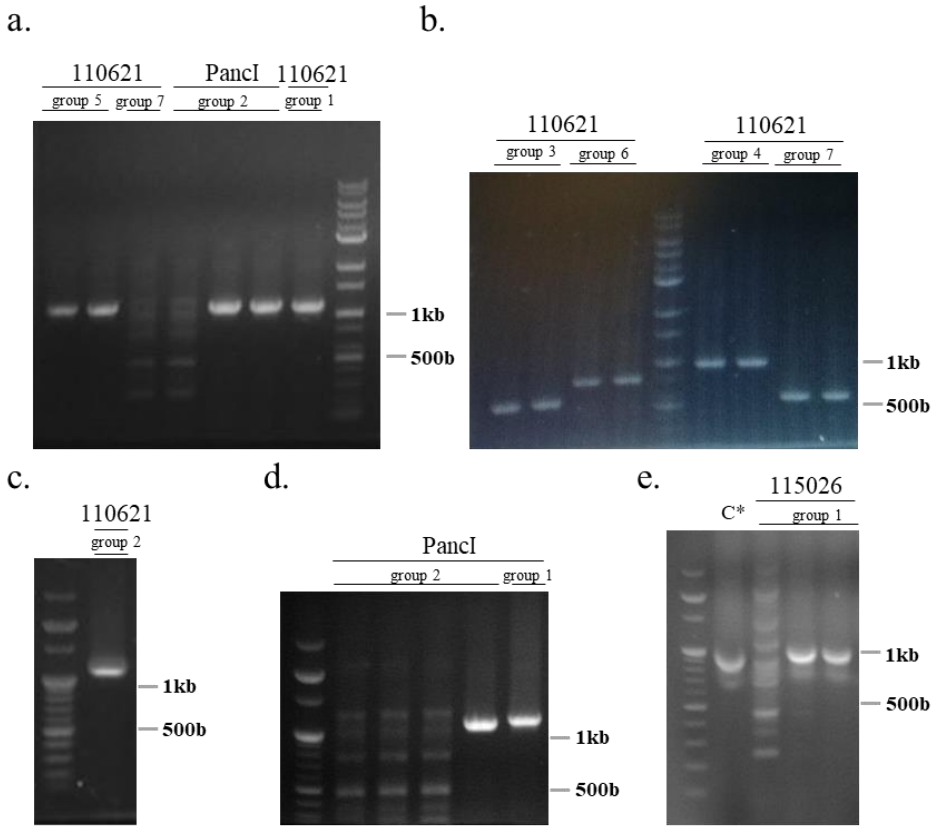
* Index: The same gene between the selected antibodies.

Supplement table 6. Summary of changes in antigen-antibody binding according to gene knockdown to find antigens

Cell name / Knockdown target gene	PANC-1	115026	19224	HEK 293T Lenti-X	HEK 293E
ANXA2	X			X	
C1QBP	X				
CALML5	★★			X	X
DSP	★★★	★★		★★★	
GJA5	★★				
GRPEL1	★★	★	★	X	★★
LARP7	★★★	★★★	★★★	★★	
MUC5AC	★★★	★★★	★★★	★★	★★
PARP-1	X				
PPP1CA	X			X	
RDX	★★★	★	X	X	
SERPINA4	★★	★	★★★	X	★★
TIA1	X				
TNKS1BP1	X				
TNKS2	X				

Supplementary figure

Supplement figure 1. The result of cloning through colony PCR.



f.

*Unit : bp

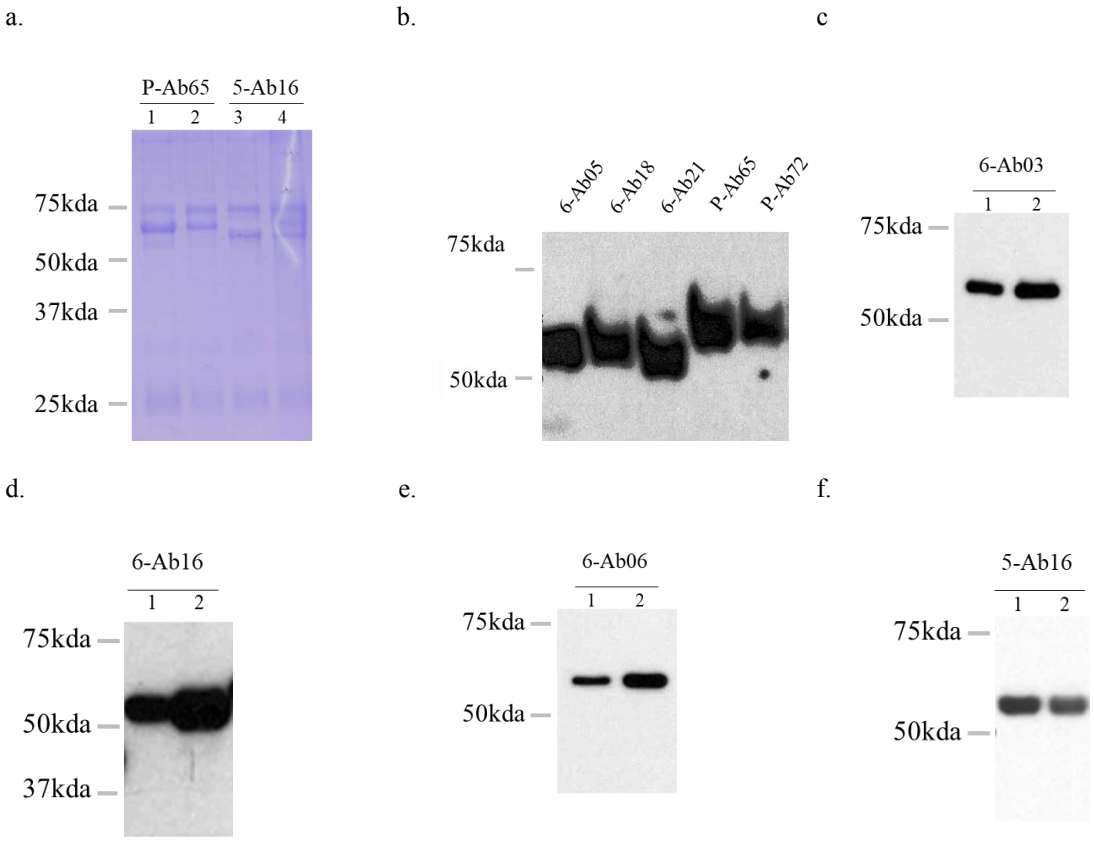
PancI	group 1	group 2
Insert size	764	764
PCR product size	≈ 1,050	≈ 1,050

115026	group 1
Insert size	755
PCR product size	≈ 1,050

110621	group 1	group 2	group 3	group 4	group 5	group 6	group 7
Insert size	761	746	770	755	776	761	782
PCR product size	≈ 1,050	≈ 1,050	≈ 500	≈ 900	≈ 1,050	≈ 700	≈ 500

*C: control for PCR condition confirmation

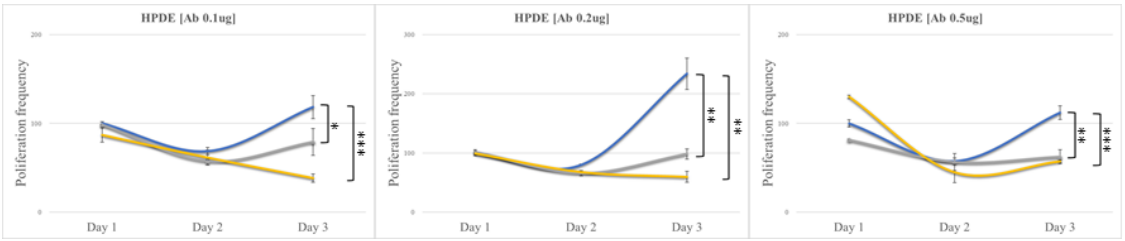
Supplement figure 2. Confirmation of expression of the selected antibody



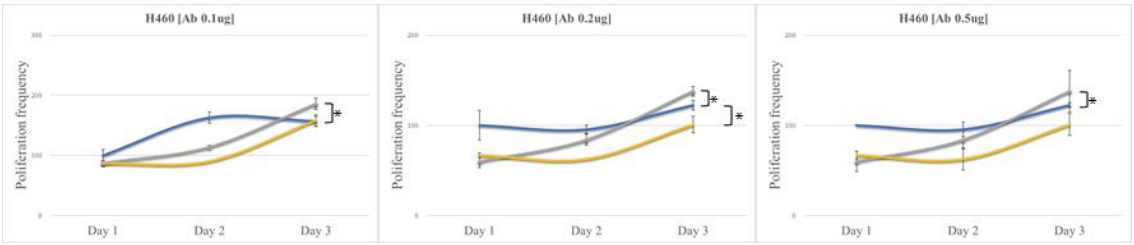
(a) The results of Coomassie staining of supernatant without serum. Partially, scFv expression was confirmed using the supernatant of P-Ab65 and 5-Ab16. 1 and 3 mean the second supernatant collected, and 2 and 4 mean the first supernatant collected. **(b ~ c)** These are the results of the western blot that confirmed the supernatant with goat anti-human IgG Fc antibody (HRP). **(b)** P-Ab65, P-Ab72, 6-Ab05, 6-Ab21 and 6-Ab18 were able to identify the band near 55kDa, which is the expression size of scFv-Fc. **(c)** 6-Ab03 was able to identify the band near 55kDa. 1 means the first supernatant collected, and 2 means the second supernatant collected. **(d)** 6-Ab16 was able to identify the band near 55kDa. 1 means the first supernatant collected, and 2 means the second supernatant collected. **(e)** 6-Ab06 was able to identify the band near 55kDa. 1 means the first supernatant collected, and 2 means the second supernatant collected. **(f)** 5-Ab16 was able to identify the band near 55kDa. 1 means the second supernatant collected, and 2 means the first supernatant collected.

Supplement figure 3. Difference in proliferation by concentration in various cells

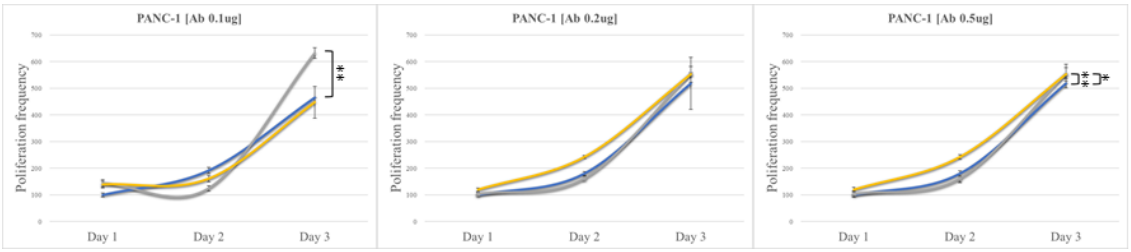
a. HPDE



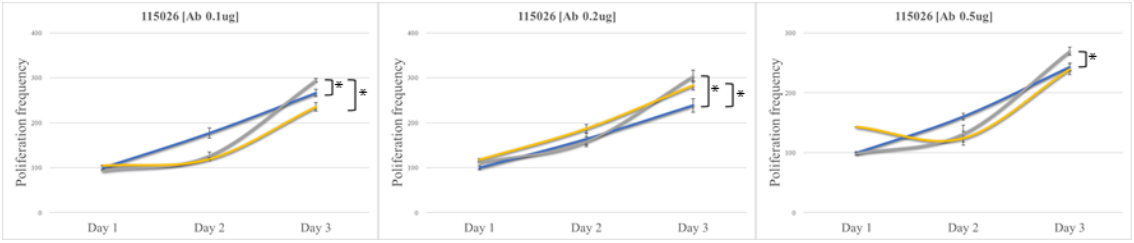
b. H460



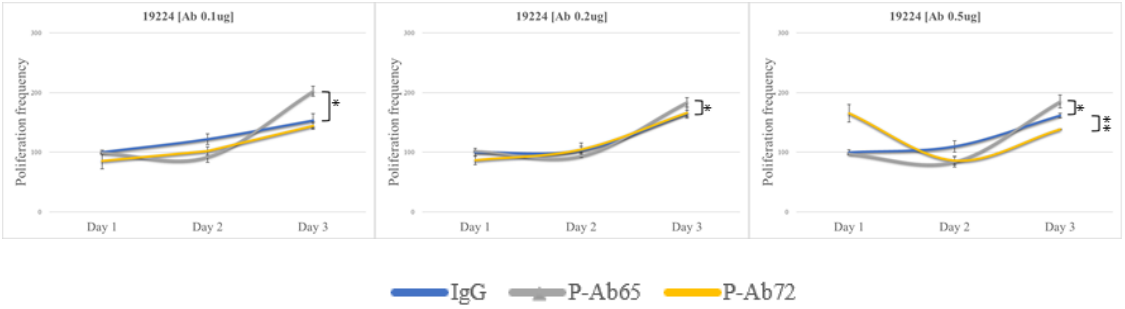
c. PANC-1



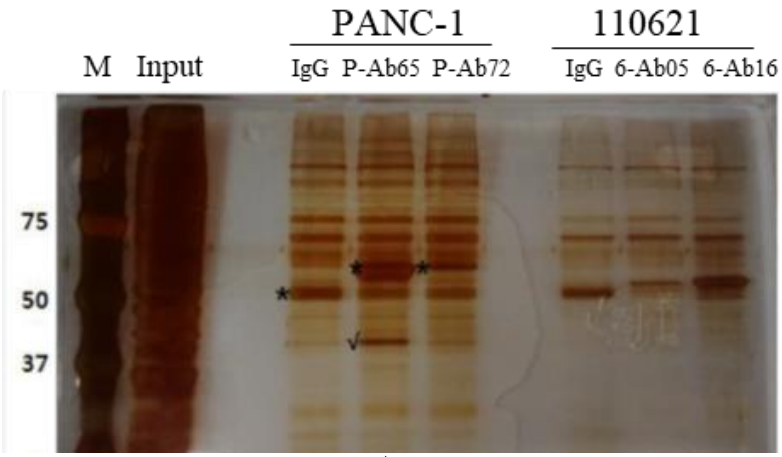
d. 115026



e. 19224

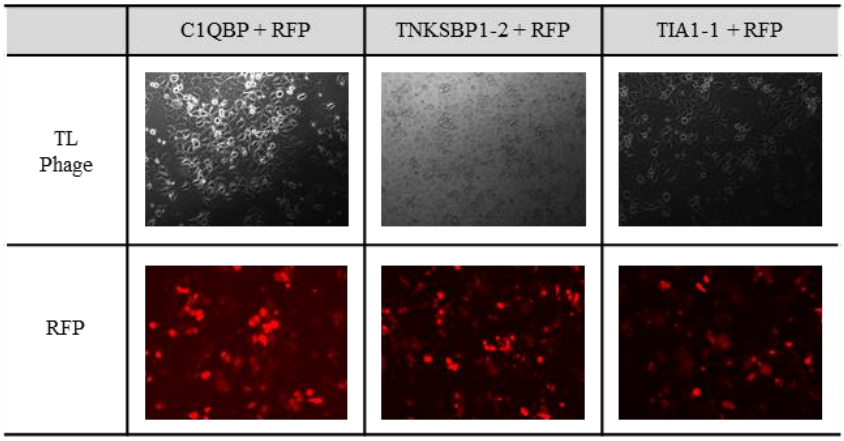


Supplement figure 4. Silver staining results before LC-MS/MS analysis for binding protein

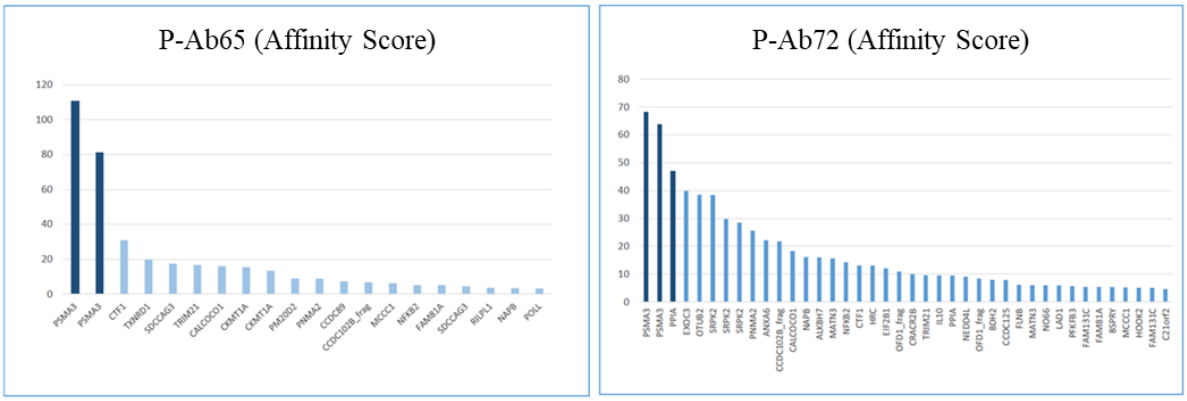


*: Antibody binding point √: Potential antigen candidate points

Supplement figure 5. Check the RFP luminescence rate using a fluorescence microscope to check the transformation efficiency (record only part of everything)

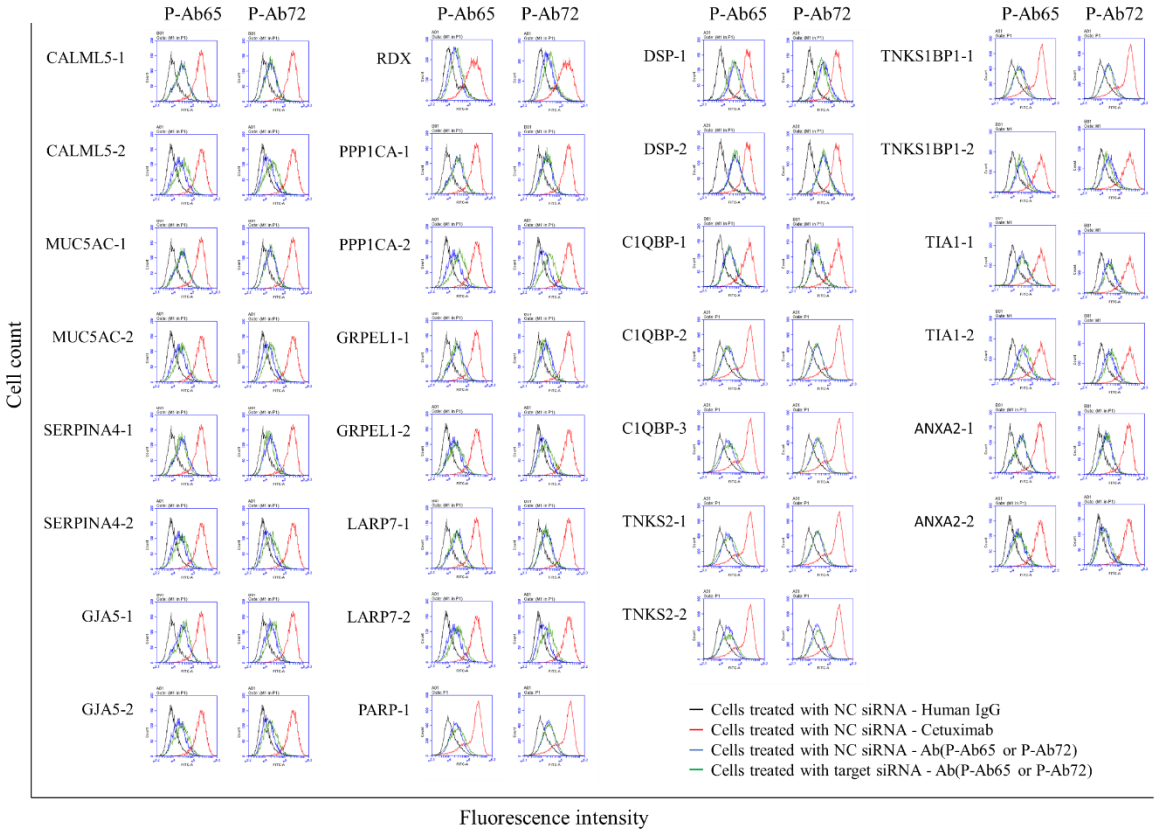


Supplement figure 6. Results of human protein microarray to find the antigen to which the selected antibody binds

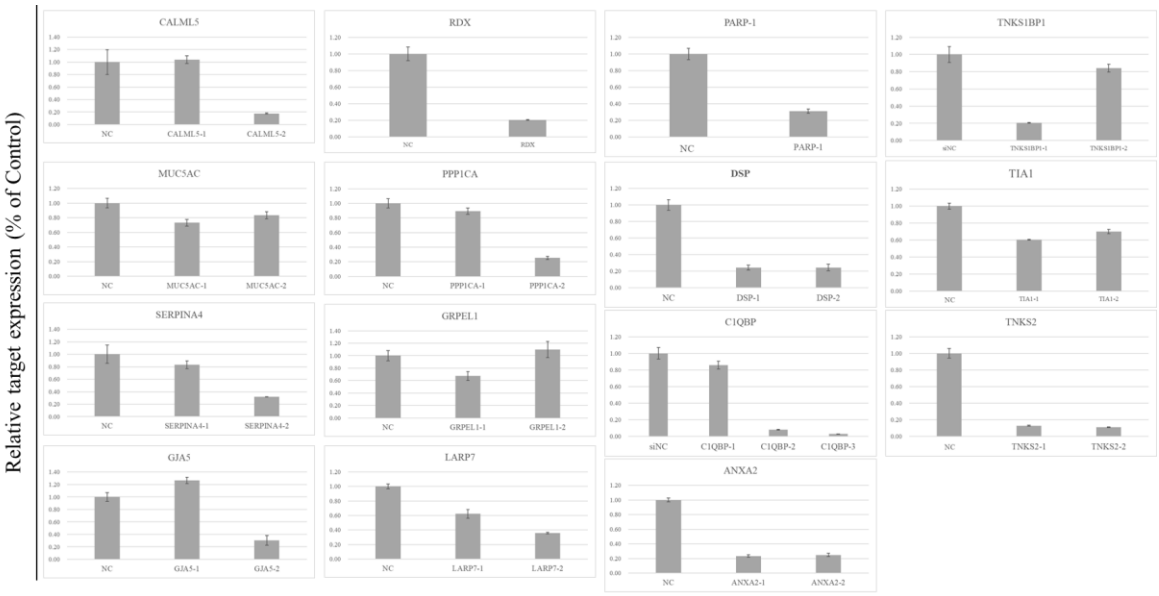


Supplement figure 7. Check knockdown and binding changes using Lipofectamine 3000 reagent.

a.



b.



Knockdown was performed using siRNA to confirm binding reduction to identify the gene of the antigen that the selected antibody is expected to bind to. (a) FACS was used to check the reduction in binding. FITC labels were attached to cells with selected antibodies (scFv-Fc) and analyzed using FACS. The black line is negative controls that reacted with human IgG to cells treated with siRNA NC. The Red line is positive control that reacted with cetuximab to cells treated with siRNA NC. The blue line is the control group that reacted with selected antibody (scFv-Fc) to cells treated with siRNA NC. The green line is the experiment group that reacted with selected antibody (scFv-Fc) to cells treated with target siRNA. (b) It shows the value of knockdown by siRNA using quantitative real-time PCR. It was analyzed based on siRNA NC.

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국문요약

췌장암은 발병 초기에는 증상이 없기 때문에 인지하기 어려움. 대부분 췌장암으로 진단된 후에는 다른 장기로 전이된 상태로 발견되기 때문에 생존율이 매우 낮음. 췌장암에 특이적으로 결합하는 항체가 발견되면 특정 암세포에 대해서만 항체-치료적 접합체를 만들어 치료에 사용할 수 있다고 가정하고 있음. 따라서, 환자유래 항체를 동정하여 종양의 특이적 항원 · 항체 결합을 통한 새로운 맞춤형 항체 치료를 만드는 것이 목표임.

항체(scFv) 라이브러리는 10 명의 췌장암 환자로부터 얻은 혈액에서 pDR-D1(파지미드 백터)을 사용하여 생성되며, 파지 디스플레이 기술을 이용하여 항체 라이브러리 스크리닝하여 암세포 표면에 특이적 결합을 하는 항체 클론을 선별함. 선택한 scFv 후보가 발현 벡터인 pDR-OriP-Fc1(포유류 카세트 벡터)에 유전자 재조합함. 293E 세포를 활용하여 scFv-Fc 를 발현시키고, 유세포 분석기를 통해 췌장암 세포에 대한 결합을확인함.

그 결과 췌장암으로부터 분리된 인간의 췌장암세포주인 PANC-1 에서 강하게 결합하는 항체를 발견함. 이외에도 선별된 항체가 유방암, 폐암, 대장암, 난소암 세포주에서도 결합되는 것을 확인함.

선별된 항체를 각 췌장암 세포에 면역침전하여 액체 크로마토그래피-질량분석법을 통해 결합되는 항원을 식별하고, 식별한 항원을 녹다운하여 항원-항체 결합의 변화를 확인하고자 짧은 간섭 RNA 를 이용하여 억제 후, 유세포 분석기를 통해 결합의 변화를 확인함. 후보 유전자 녹다운 후 항원-항체 결합의 차이는 여러 번 확인되었고, 이를 통해 선택된 항체의 항원 후보가 결정됨. 항원의 가장 가능성이 높은 유전자는 MUC5AC, LARP7, DSP, SERPINA4 및 GRPEL1 임.

선택된 항체가 결합하는 항원으로 확인되면 환자유래이종이식(PDX) 모델에 항체 선별항체를 단일 투여하거나 선별항체와 면역치료제를 동시투여(조합 면역요법)하여 암 치료에 효과 기대함. 우리 연구는 췌장암 환자로부터 추출된 항체가 췌장암 치료제로 사용될 수 있음을 시사함.

핵심 키워드: 췌장암, 파지 디스플레이, 항체 라이브러리, 면역요법, scFv, 세포 패닝

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먼저 힘들었던 시기에 꿈에 한 발짝 다가오도록 지원해주시고 격려해주신 장수환 교수님께 진심으로 감사합니다. 교수님 덕분에 시도해볼 수 있었고 교수님의 지도로 연구가 무엇인지, 어떤 방향으로 나아가야 하는지를 알 수 있었습니다. 저의 더딘 배움에도 저를 응원해주시고, 가르침을 주신 교수님이 있어 지금의 제가 있을 수 있었습니다.

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마지막으로 저를 믿어 주시고 학업에 전념하도록 도와준 부모님께 감사합니다. 갑자기 대학원 진학하겠다고 말해서 놀라셨겠지요. 그럼에도 저를 이해해주시고 응원해 주셔서 여기까지 할 수 있었습니다. 항상 감사하고 사랑합니다.

이외에도 여기에 미처 적지 못한 많은 분들께 감사합니다. 보이는 곳에서 혹은 보이지 않는 곳에서 응원해준 분들이 있어 제가 있을 수 있었습니다.

짧은 기간 동안 췌장암을 연구하면서 연구자분들의 노력이 보이는 것에 비해 얼마나 힘들고 어려운 일인지 알게 되었습니다. 아직 췌장암의 치료는 해결해야 할 난관이 많습니다. 그분들의 노고에 진심으로 감사드리며 한 명의 연구자가 되어 항상 응원하겠습니다.

짧은 시간이었지만 의미 있는 시간이었습니다. 도움을 주신 모든 분들께 감사의 마음을 전하며, 항상 행복하고 건강하길 기원합니다.

안 세 희 드림.