

# An Epithelial Cell Adhesion Molecule- and CD3-Bispecific Antibody Plus Activated T-Cells Can Eradicate Chemoresistant Cancer Stem-like Pancreatic Carcinoma Cells *In Vitro*

MASAYO UMEBAYASHI<sup>1</sup>, AKIFUMI KİYOTA<sup>1</sup>, NORIHIRO KOYA<sup>1</sup>, HIROTO TANAKA<sup>1</sup>,  
HIDEYA ONISHI<sup>2</sup>, MITSUO KATANO<sup>2</sup> and TAKASHI MORISAKI<sup>1</sup>

<sup>1</sup>Fukuoka General Cancer Clinic, Fukuoka, Japan;

<sup>2</sup>Department of Cancer Therapy and Research,  
Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

**Abstract.** Cancer stem-like properties of various types of cancer, including pancreatic cancer, one of the most aggressive types, correlate with metastasis, invasion, and therapeutic resistance. More importantly, chemoresistance in cancer stem-like cells (CSLCs) is a critical problem for eradication of pancreatic cancer. Several cell surface markers, such as CD44 and epithelial cell adhesion molecule (EpCAM), are molecular targets on CSLCs of pancreatic carcinoma. In this study, we investigated whether catumaxomab, a clinical-grade bi-specific antibody that binds to both EpCAM on tumor cells and CD3 on T-cells, combined with activated T-cells can eliminate chemoresistant pancreatic CSLCs *in vitro*. Firstly, we established a CSLC line (MU-PK1) from human pancreatic carcinoma cells derived from a patient with chemoresistant and disseminated pancreatic cancer. These CSLCs were almost completely resistant to gemcitabine-mediated cytotoxicity up to a concentration of 10 µg/ml. The cells expressed high levels of CSLC markers (CD44 and EpCAM) and had significantly higher capacities for sphere formation, invasion, and aldehyde dehydrogenase-1 expression, which are associated with cancer stemness properties. We found that pre-treatment with catumaxomab and subsequent addition of interleukin-2/OKT3 activated autologous T-cells eliminated CSLCs during a short incubation period. Moreover, when MU-PK1 cells were cultured under hypoxic conditions, the CSLCs became more

aggressive. However, the combination of cytokine-activated killer T-cells with catumaxomab successfully lysed almost all these cells. In conclusion, catumaxomab combined with activated T-cells may be a potent therapeutic modality to eradicate chemoresistant pancreatic CSLCs.

The overall survival time of patients with pancreatic cancer has recently been significantly prolonged by advances in various types of therapies including gemcitabine treatment, but this disease still remains lethal (1). One of the reasons for the difficulties in curing pancreatic cancer is the existence of therapy-resistant cancer cells (2).

It is now widely accepted that most tumours contain a small subpopulation of cells with stem cell-like properties which have the ability to generate diverse mature cells types by differentiation (3). These cells, termed cancer stem cell-like cells (CSLCs), have the ability to produce all of the distinct cell types found in their original tumour (4). CSLCs are associated with higher rates of metastasis and poor patient prognoses, and more clinically relevant, are resistant against various therapies (5).

There is no established therapeutic option for tumours with cancer stem cell-like properties. One of the properties associated with cancer stem cells (CSCs) is drug resistance (5). Conversely, chemoresistant tumour cells have been shown to possess CSC-like properties (6, 7). Dallas *et al.* reported that chemoresistant colorectal cancer cells possess stem-like properties, such as a sphere-forming ability and high expression of CSC-like surface markers CD44/CD133 (6). Therefore, development of chemoresistant cell lines from cancer cells may be useful to fundamentally establish a therapeutic model for CSLCs.

One approach to overcome the chemoresistance of CSLCs is identification of cell surface molecules for therapeutic targeting. There are several known surface markers for CSLCs. Indeed, recent findings have shown that antibodies

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Correspondence to: Dr. Takashi Morisaki, Fukuoka General Cancer Clinic, 3-1-1 Sumiyoshi, Hakata-ku, Fukuoka 812-0018, Japan. Tel: +81 922827696, Fax: +81 924056376, e-mail: tmorisaki@cancer-clinic.jp

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targeting CSCs may be useful for cancer eradication (8). For example, epithelial cell adhesion molecule (EpCAM), a marker that distinguishes CSLCs, is overexpressed in many types of human cancers, including pancreatic cancer and cholangiocarcinoma, and is associated with poor prognosis (9, 10). Accumulating evidence suggests that EpCAM-targeting therapies can inhibit various types of cancer (11, 12). Thus far, catumaxomab, a bi-specific antibody that binds to EpCAM on tumour cells and CD3 on T-cells for subsequent activation, has been approved for clinical use to treat malignant ascites.

Tumour cells can be lysed by activated T-cells to a greater degree than that by resting T-cells because they express more activating surface and cytotoxic molecules. The cytotoxicity of activated T-cells is at least partly dependent on NKG2D/DNAM1 systems (13, 14). Activated and expanded CD8<sup>+</sup> T-cells can lyse tumour cells through the NKG2D system independently of the class I TcR system (13). Moreover, cytokine-activated killer T (CAT)-cells have shown clinical benefits when used for adoptive immunotherapy (15). CAT cells can be expanded *ex vivo* in the presence of a low dose of recombinant human interleukin-2 (IL-2) and an anti-CD3 agonistic antibody (OKT3). Therefore, CAT cells combined with a tumour-targeting agonistic antibody to CD3 can efficiently eliminate CSLCs.

In the present study, we established an immunotherapy model for eradication of pancreatic CSLCs. We examined whether the combination of a bi-specific antibody and activated T-cells can eradicate pancreatic CSLCs. Our results provide rational evidence for a possible therapeutic strategy in the treatment of chemoresistant pancreatic cancer.

## Materials and Methods

**Reagents.** The humanized mouse trifunctional antibody to human EpCAM, catumaxomab (Removab<sup>TM</sup>), was purchased from Fresenius Biotech GmbH (Graefelfing, Germany). For cell imaging, calcein-AM was purchased from Dojindo (Osaka, Japan). Gemcitabine was purchased from Eli Lilly (Indianapolis, IN, USA).

**Establishment and characterization of a pancreatic carcinoma cell line from a patient with chemorefractory metastatic pancreatic cancer and ethics statement.** Peripheral blood mononuclear cells (PBMCs) and peritoneal fluid were obtained from a 48-year-old male patient with metastatic pancreatic cancer, who had undergone chemotherapy consisting of gemcitabine and TS-1, and whose disease then progressed to the formation malignant ascites with chemoresistance. The patient's PBMCs from leukapheresis for adoptive immunotherapy were cryopreserved at -80°C until use. Cells in the peritoneal fluid were cultured in DMEM/F12 (Life Technologies, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA). After establishment of the cell line, the cells were characterised by surface antigens and gemcitabine sensitivity. *In vitro* cytotoxicity assays were also performed as described below.

Written informed consent was obtained from the patient. This study was approved by the Ethical Committee from the Fukuoka General Cancer Clinic.

**Monoclonal antibodies and flow cytometry.** Surface markers of tumour cells were labelled by direct or indirect immunofluorescence using the following monoclonal antibodies: anti-CD44-PE (Immunotech Beckman Coulter, Brea, CA, USA), anti-EpCAM-FITC (BioLegend, San Diego, CA, USA), anti-MICA/B-PE (R&D Systems, Minneapolis, MN, USA), anti-ULBP-1 (R&D Systems), anti-ULBP-3 (R&D Systems), anti-HLA-ABC-FITC (Beckman Coulter), anti-CD24 (Beckman Coulter), anti-CD133 (Miltenyi Biotec GmbH) and goat anti-mouse IgG-Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). To detect CSC-like markers, detached cells were washed twice in cold phosphate-buffered saline and stained with mouse anti-human PE-labelled anti-CD44 and FITC-labelled anti-EpCAM for 60 min at 4°C. Surface markers on CAT cells were labelled by direct or indirect immunofluorescence using anti-CD3-FITC, anti-CD16-FITC, anti-CD56-PE (Immunotech Beckman Coulter), and anti-NKG2D-PE (R&D Systems) monoclonal antibodies for 60 min at 4°C. Fluorescence was detected using an FC500 flow cytometer (Beckman Coulter) and expressed as the relative mean fluorescence intensity (MFI) or percentage of positive cells.

**Cell proliferation assay.** Cells were treated with different concentrations of gemcitabine, catumaxomab, or with dimethyl sulphoxide (DMSO) as the vehicle control. DMSO concentrations were <0.1%. For proliferation assays, cells were cultured in 96-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) at 5×10<sup>4</sup> cells/ml in 100 µl of medium. Assays were performed over 48 h with a minimum of five replicates. For drug treatments, cells were treated with different concentrations of catumaxomab (0.1 pg/ml to 10 ng/ml) or gemcitabine (0.01 to 100 µg/ml). Proliferation assays were performed using a WST-8 cell proliferation kit (Dojindo, Osaka, Japan) according to the manufacturer's instructions.

**Aldefluor assay.** Aldehyde dehydrogenase (ALDH) activity in viable cells was determined using a fluorogenic dye-based Aldefluor assay (Stem Cell Technologies, Grenoble, France) according to the manufacturer's instructions. A total of 1×10<sup>6</sup> cells/ml were suspended in Aldefluor assay buffer containing ALDH substrate (bodipy-aminoacetaldehyde) and incubated for 20 min at 37°C. As a reference control, cells were suspended in buffer containing bodipy-aminoacetaldehyde in the presence of diethylaminobenzaldehyde (DEAB), a specific ALDH1 inhibitor. Brightly fluorescent ALDH-expressing cells (ALDH1<sup>high</sup>) were detected in the green fluorescence channel (520-540 nm) of the FC500 flow cytometer.

**Invasion assay.** Cells (5×10<sup>4</sup>) were seeded into the upper compartments of BD BioCoat<sup>TM</sup> Matrigel Invasion Chambers (BD Bioscience, San Jose, CA, USA), and DMEM supplemented with 10% foetal bovine serum was added to the lower compartment according to the manufacturer's instructions. The invasion chamber was incubated for 24 h under standard culture conditions. After incubation, the non-invading cells were removed from the upper surface of the membrane by gentle scrubbing, and the cells on the lower surface of the membrane were stained with crystal violet. Cell counting was facilitated by photographing the membrane under a microscope at ×200 magnification and counting the cells in five fields per membrane of triplicate membranes.

**Sphere formation assay.** The sphere formation assay was performed as described elsewhere with modification (6). Briefly, cells were seeded in six-well, ultralow, attachment plates (Corning, NY, USA) at a density of 1,000 cells/ml in DMEM/F12 containing 20 ng/ml human basic fibroblast growth factor and 100 ng/ml epidermal growth factor (R&D systems), and incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The cells were passaged after 7-10 days. Morphological changes were observed daily under an inverted light microscope for 14 days. Cell clusters over 50 µm were counted in each well.

**Generation of ex vivo expanded and activated lymphocytes.** To prepare effectors cells, CAT cells were induced from PBMCs obtained from healthy volunteers or a patient with written informed consent. PBMCs were treated with a low concentration of recombinant IL2 (200 U/ml; Chiron, Emeryville, CA, USA) and 5 µg/ml Orthoclone® (OKT3; Janssen-Cilag, Tokyo, Japan), and expanded for 10-14 days to obtain sufficient numbers of activated lymphocytes, including mainly T-cells and a very small number of natural killer cells.

**Cytotoxicity assay.** To measure the cytotoxicity of CAT cells, we modified an adherent target detachment (ATD) assay that has been described previously (16, 17). Target cells (5×10<sup>3</sup> per well) were seeded in a flat-bottom 96-well plate and incubated overnight to allow adherence. Different concentrations of catumaxomab were added to the target cells, followed by preincubation for 48 h. The cells were then washed and cultured with or without activated T-cells (5-10×10<sup>4</sup> cells/well) for a further 4 h. Effectors cells and dead target cells that detached from the culture surface were removed by washing. To quantify viable adherent cells, WST-8 reagent solution (from the WST-8 cell proliferation kit) was added to the washed wells, followed by incubation for 1 h at 37°C. The absorbance at 450 nm was then measured using a microplate reader (ImmunoMini NJ-2300; Nalge Nunc International, Panorama Creek Drive Rochester, Nk, USA). Detached cells were stained with 7-amino-actinomycin D (BD Bioscience) to confirm that the detached tumour cells were non-viable. These experiments were performed under normoxic (20% O<sub>2</sub>) or mildly hypoxic (7% O<sub>2</sub>) conditions.

**Calcein-release cytotoxicity assay and cell imaging.** Antibody-mediated cellular cytotoxicity (ADCC) assays were performed by calcein-AM release. Briefly, target cells were resuspended at 1×10<sup>5</sup> cells/ml in complete medium and allowed to adhere in a 96-well plate overnight. Then the cells were incubated at 37°C for 48 h in the presence or absence of catumaxomab at different concentrations. After incubation, the cells were washed with medium, incubated with calcein-AM for 30 min, and then washed before CAT cells (ET=40:1) were added to the cultures. After 4 h of incubation at 37°C, the plate was washed and the adherent cells were analyzed under a fluorescence microscope (IX81; Olympus, Tokyo, Japan) by Lumina Vision software (version 2.4.2; Mitani, Fukui, Japan). Images were captured with a colour CCD camera (DPI72; Olympus) and LUC plan FLN objective lens (Olympus). All procedures were performed at 20-25 °C.

**Statistical analysis.** All data are expressed as the mean±standard error of the mean (SEM) unless indicated otherwise. Differences between groups were assessed for statistical significance using the Mann-Whitney test or paired Student's *t*-test depending on the data distribution. A value of *p*<0.05 was considered to indicate statistical significance.

## Results

**Characterization of the CSC-like MU-PK1 cell line.** In the present study, we established a cell line resembling CSLCs from a patient with metastatic and disseminated pancreatic carcinoma. We then investigated whether the CSLCs could be eliminated by catumaxomab-plus-autologous activated T-cells. The pancreatic carcinoma cell line (MU-PK1) was generated from the primary culture as described in the Materials and Methods section. Firstly, the cells were thoroughly examined in terms of their various stem-like properties. As shown in Figure 1C and D, the cells displayed an elongated and irregular spindle shape, suggesting that MU-PK1 cells had undergone epithelial-mesenchymal transition (EMT) as reported previously (18). To confirm other CSC-like properties of the MU-PK1 cells, we investigated their sphere-forming capacity, invasive capacity, and ALDH1 activity. CSCs in suspension have the ability to form spheres in the absence of serum (19). We, therefore, firstly evaluated the ability of the cell line to form spheres under serum-free conditions. The sphere-forming ability was quantified by plating a limited number of cells in each well of a low-attachment 96-well plate. As a result, we observed cell sphere formation, although the degree of sphere-forming capacity of MU-PK1 cells could not be assessed because of the lack of a control (Figure 2A). Both invasion and ALDH1 activity were detected (Figure 2B and C), although the ALDH1 activity was low. Moreover, the cell line was highly resistant to gemcitabine up to a concentration of 10-100 µg/ml (Figure 2D). Considering that the IC<sub>50</sub> of gemcitabine in human pancreatic carcinoma cell lines is 0.1-10 µg/ml (20), the cell line appeared to be highly resistant against gemcitabine. We next characterized the pancreatic cancer cell line by focusing on cell surface markers. The cells expressed low levels of stress antigens (MICA/B, ULBP-1, and ULBP-3) and high levels of stem-like markers CD44/EpCAM (Figure 3). Collectively, these data demonstrated that the MU-PK1 cell line had CSC-like properties.

**Killing of MU-PK1 cells by cytokine activated T-cells alone and in combination with catumaxomab.** Next, we performed *in vitro* cytotoxicity assays using the cell line as the target and autologous activated T-cells as effectors. The activated lymphocytes were CD3<sup>+</sup>/NKG2D<sup>high</sup> T-cells as shown in Figure 4A, but the cytotoxic activity against the autologous tumour cells was relatively low (Figure 4B and, C). EpCAM is associated with cancer cell signalling and stem-like properties (10, 11). Therefore, we chose EpCAM as a cell surface target for immunotherapy of CSLCs. Because catumaxomab is a tri-functional monoclonal antibody with a functional Fc domain and bispecific antigen-binding sites for EpCAM on epithelial tumour cells and CD3 on T-cells, we determined whether catumaxomab alone or in combination with T-cells could mediate cytotoxic activity against MU-



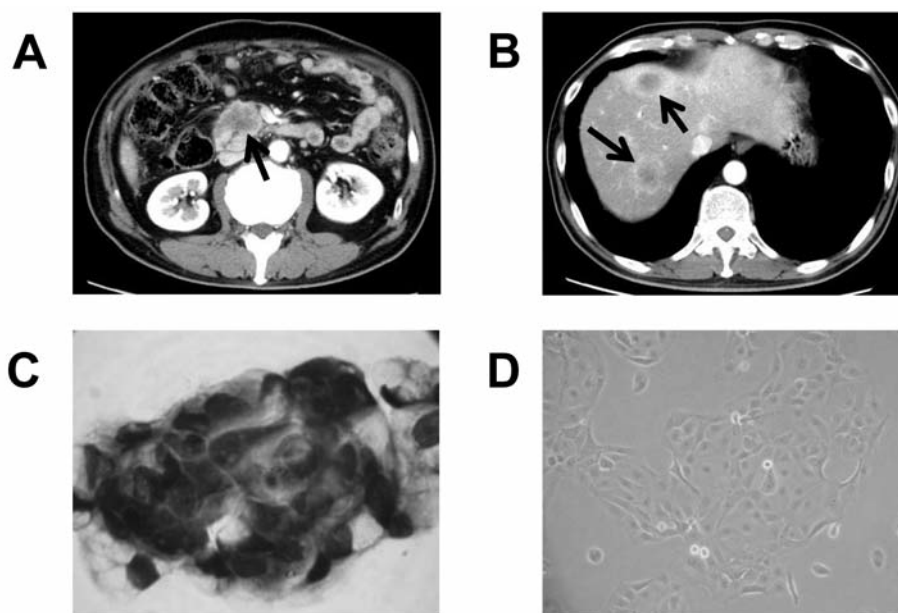


Figure 1. Clinical and cytological findings of the patient. Upper left, computed tomography of the original tumour (arrow); upper right, computed tomography of the liver metastasis (arrow); lower left, Giemsa staining of cells from malignant ascites; lower right, microscopic observation of the established cell line.

PK1 cells. Although the concentration of catumaxomab alone was increased from 0.0001 ng/ml to 10 ng/ml, there was no inhibition of cell growth in the cell line (data not shown). Therefore, we next determined whether the combination of catumaxomab and autologous CATs could induce cytotoxic activity against CSLCs *via* ADCC by comparing CATs alone and in combination with catumaxomab as effector cells in an calcein-release assay. CATs were obtained by culturing resting PBMCs in the presence of low-dose IL2 and OKT3 for 10-14 days. Activated T-cells from the patient mainly consisted of CD3<sup>+</sup>/NKG2D<sup>high</sup> T-cells, with much higher expression of NKG2D, which is a potent co-stimulatory molecule, than that of resting lymphocytes (Figure 4A). The combinator of CATs plus catumaxomab led to a significant increase in cytotoxicity against the tumour cells. Although the cytotoxicity was modest for CATs alone, addition of 1 ng/ml catumaxomab significantly augmented the cytotoxicity of CATs (Figure 5). As shown in Figure 5, the augmentation of CAT cell-mediated cytotoxicity by catumaxomab was confirmed in the image analysis by Lumina Vision, which showed a significant reduction of calcein release from tumour cells incubated with CATs and catumaxomab. These results indicate that the cytotoxicity of the combination of catumaxomab and CAT cells was significantly increased compared to that of CATs alone, confirming catumaxomab-mediated ADCC. We further investigated the time dependency of catumaxomab-induced

CAT-mediated cytotoxicity against CSLCs. When subjected to ADCC testing at increasing doses of catumaxomab, we found that doses of 0.1-10 ng/ml catumaxomab significantly increased the susceptibility of both the parental cells and CSLCs to cytotoxicity induced by CAT cells. These effects reached a plateau at 10 ng/ml catumaxomab (data not shown). When the catumaxomab dose was fixed at 10 ng/ml, incubation with CATs for 4-24 h was sufficient for complete eradication of the tumour cells (Figure 6). Incubation with 10 ng/ml catumaxomab and CAT cells for 24 h resulted in almost complete tumour cell lysis ( $p=0.01$ ) (Figure 6). In the presence of catumaxomab for 24 h, tumour cells were detached from the bottom of the well, surrounded by CATs, and showed apoptotic changes (Figure 6, lower panel).

*Characterization of hypoxia-treated MU-PK1 cell sub-cell line and killing of those cells by activated T-cells and catumaxomab.* Hypoxic conditions may exacerbate CSC-like properties. MU-PK1 cells were cultured under hypoxic conditions (7% O<sub>2</sub>) for several weeks, and the resulting cell line was termed MK-PK1-hypoxia. We first compared gemcitabine sensitivities of the two cell lines. The invasive capacity and expression of CD44 and EpCAM in MK-PK1-hypoxia cells were slightly increased compared with those of the parental cells (Figure 7A and B). Next, the cells were treated with different concentrations of gemcitabine (0, 0.01, 0.1, 1, and 10 µg/ml) for 48 h, and then cell viability was evaluated by the WST-8 assay. The results

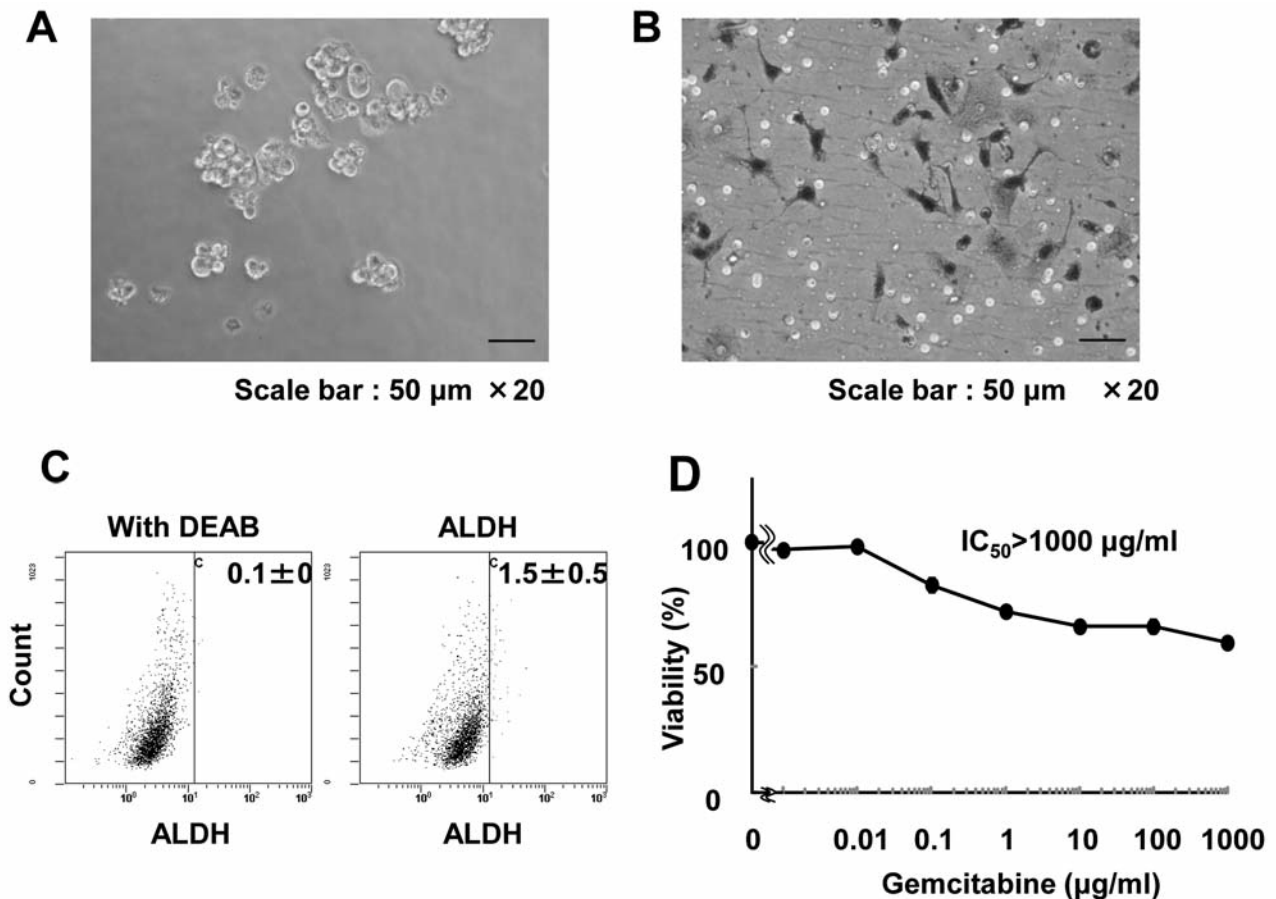


Figure 2. Characterization of the cancer stem-like properties of MU-PK1 cells. A: Sphere-forming capacity. The cells were evaluated for their sphere-forming capacity *in vitro*. A total of  $2 \times 10^3$  cells were plated in serum-free medium. At approximately 14 days after seeding, spheres were quantified by counting the number of clusters of sphere-forming cells. B: Invasiveness of the cell line. A representative insert from the invasion assays is shown. Membranes coated with Matrigel in cell culture inserts were used for invasion assays. For each insert, cells in five fields were counted under a microscope ( $\times 200$ ). Image shows crystal violet-stained invading cells. C: ALDH expression in the cells. An ALDEFLUOR assay was used to identify cells with high ALDH1 expression (ALDH<sup>+</sup>). The ALDH inhibitor DEAB was used for the negative control (left panel). Cells without the inhibitor, for which the pattern shifted to the right, were considered as ALDH<sup>+</sup> cells (right panel). D: Effect of gemcitabine on the growth of the cell line. Cells were treated with gemcitabine at different concentrations for 48 h. For each concentration, five replicates were analyzed in a single experiment, and representative experimental data from three experiments are shown.

showed that MU-PK1-hypoxia cells were relatively resistant to gemcitabine (Figure 7C). We further compared the sensitivities of the parental cell line and MU-PK1-hypoxia cells to catumaxomab-mediated T-cell cytotoxicity. As shown in Figure 8, the sensitivity to catumaxomab-mediated CAT-dependent cytotoxic activity was similar in both MU-PK1-hypoxia and parental cells.

## Discussion

We established a pancreatic carcinoma cell line termed MU-PK1, which possesses CD44<sup>high</sup> and EpCAM<sup>high</sup> surface markers associated with CSC-like properties such as a

sphere-forming capacity, and exhibited an EMT-like morphology and strong resistance to gemcitabine. Using this cell line, we demonstrated that catumaxomab in combination with activated T-cells can eradicate pancreatic CSLCs *in vitro*. These results collectively show that catumaxomab when combined with activated T-cells may be a novel immunotherapy for eradication of pancreatic CSLCs expressing EpCAM.

We first successfully established chemoresistant CSLCs and then identified cell surface molecules for their targeting and eradication. We found that these cells exhibited strong CSC-like properties such as CD44/EpCAM expression and ALDH1 activity. The cells were resistant to gemcitabine up

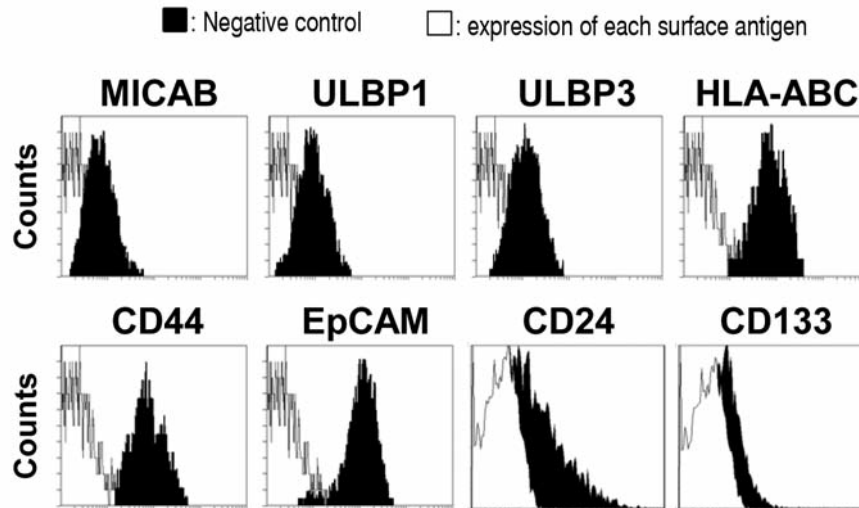


Figure 3. Flow cytometric analysis of cell surface antigens MICA/B, ULBP1, ULBP3, HLA-ABC, CD44, CD24, CD133, and EpCAM.

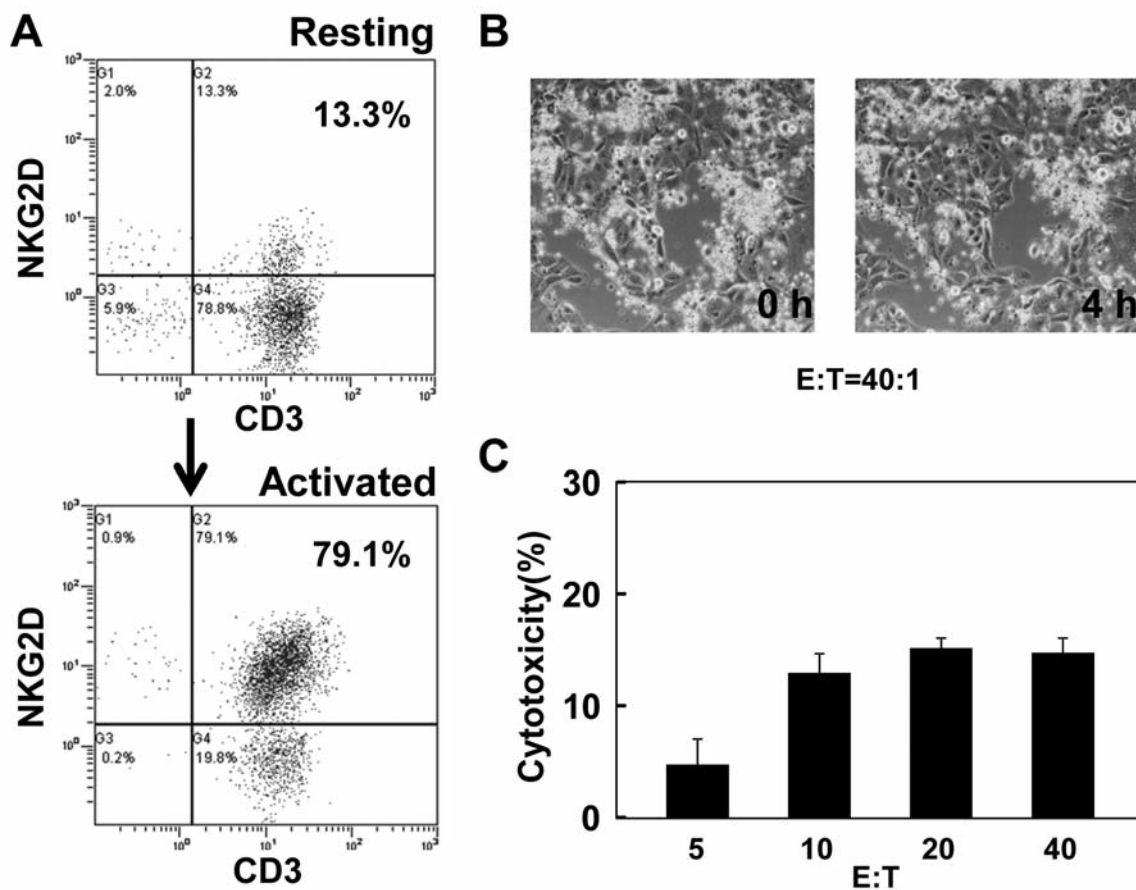


Figure 4. Flow cytometric analysis of autologous activated lymphocytes from the patient and killing of MU-PK1 cells. A: The percentage of CD3/NKG2D-positive cells is shown. B: Images of the tumour cells in an adherent target-detachment (ATD) assay. Left, control (before treatment with CAT cells); right, 4 h after the addition of activated T-cells. C: Cytotoxicity data in (B) analyzed by ATD assay, as described in the Materials and Methods.

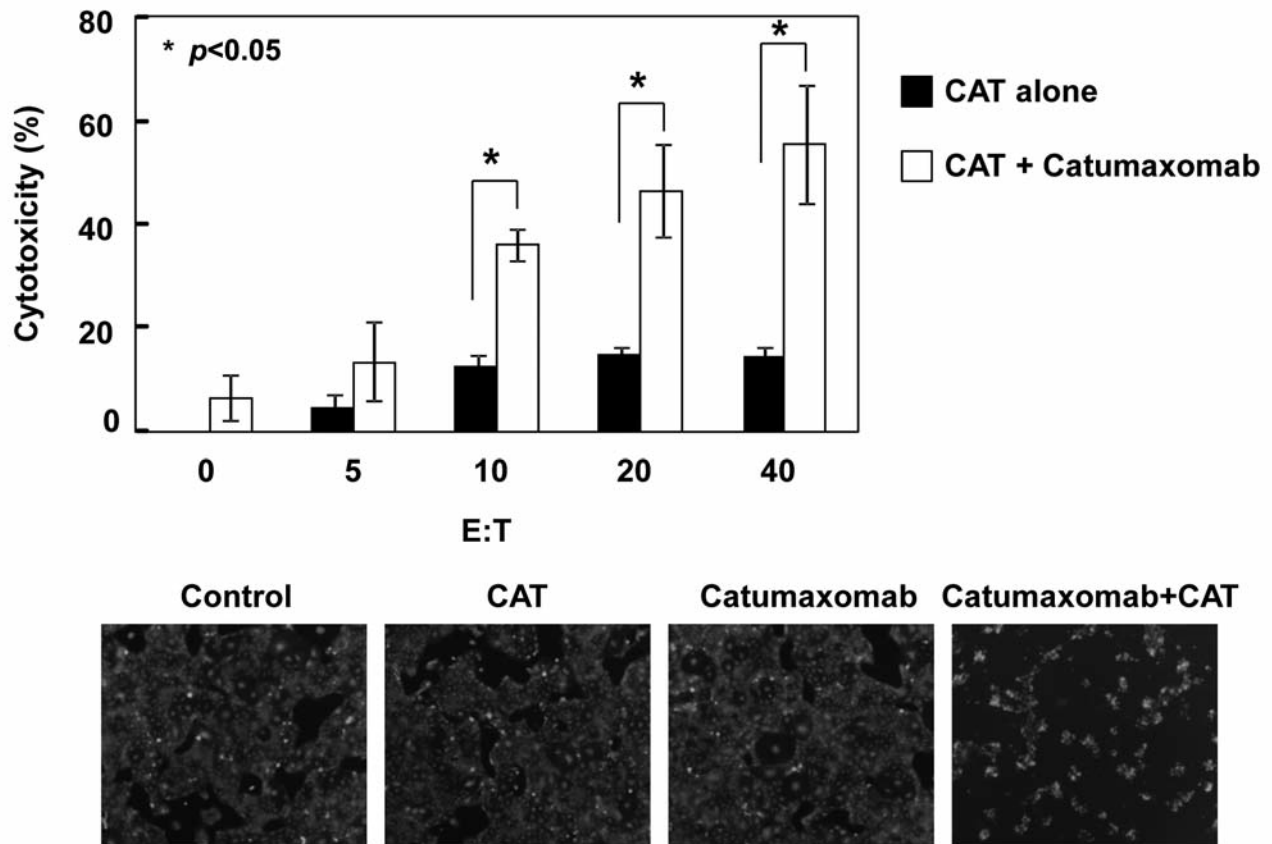


Figure 5. Killing of MU-PK1 cells by autologous CATs alone and in combination with catumaxomab. A: Cytotoxic activity of CAT cells against MU-PK1 cells with/without catumaxomab pre-treatment. A calcein-release assay was used (as described in the Materials and Methods) to detect the cytotoxicity of CATs against MU-PK1+hypoxia cells following pre-treatment with catumaxomab (10 ng/ml). The ratios of effector CATs to target MU-PK1 cells were approximately 5:1, 10:1, 20:1, and 40:1. The incubation time was 4 h. All data are expressed as the mean percentage of specific cytotoxicity $\pm$ SEM. Data are from triplicates and representative of three independent experiments. \* $p<0.05$ . B: Imaging of cytotoxicity toward MU-PK1 cells by CAT cells with catumaxomab. MU-PK1 cells were pretreated with catumaxomab (10 ng/ml), washed, and then stained with calcein-AM. Subsequently, the MU-PK1 cells were incubated with CATs for 4 h and then washed. The cells were observed by fluorescence microscopy, and the acquired images were analyzed to quantify the cytotoxic activity with Lumina Vision as described in the Materials and Methods.

to a concentration of 10-100  $\mu$ g/ml. Because the mechanisms by which tumours acquire chemoresistance have been attributed to the appearance of CSLCs, our results confirmed the establishment of a CSLC line.

Indeed, many studies have demonstrated that CSLCs are chemoresistant and have distinct cell surface markers. The chemoresistant cells were highly invasive, and showed an increased ability to form spheres in serum-free medium, which are properties consistent with the CSC phenotype. These properties were observed for more than 12 months. Thus, we successfully established CSLCs from human pancreatic carcinoma cells.

Targeting CSLCs therapeutically is important and likely to be challenging. Because CSLCs are molecularly distinct from bulk tumour cells, targeting their molecular differences

is an attractive strategy. For example, cell surface marker expression can be used for antibody-directed therapy to target proteins such as CD44 and EpCAM. Conventional chemotherapeutic drugs require penetration into cells, and drug resistance mechanisms such as expression of ATP-binding cassette transporters may eventually occur. Therefore, targeting molecules on the cell surface may be important to circumvent the drug resistances of CSLCs.

EpCAM has been characterised as a tumour-associated antigen (10, 11). Recent studies of EpCAM have focused on its role in carcinogenesis (10). Among the identified CSC-like markers, EpCAM is an excellent target for immunotherapy because it is specifically expressed in epithelial tumour cells, abundantly expressed in various types of CSLCs, presented at the cell surface without being released into circulation, and is



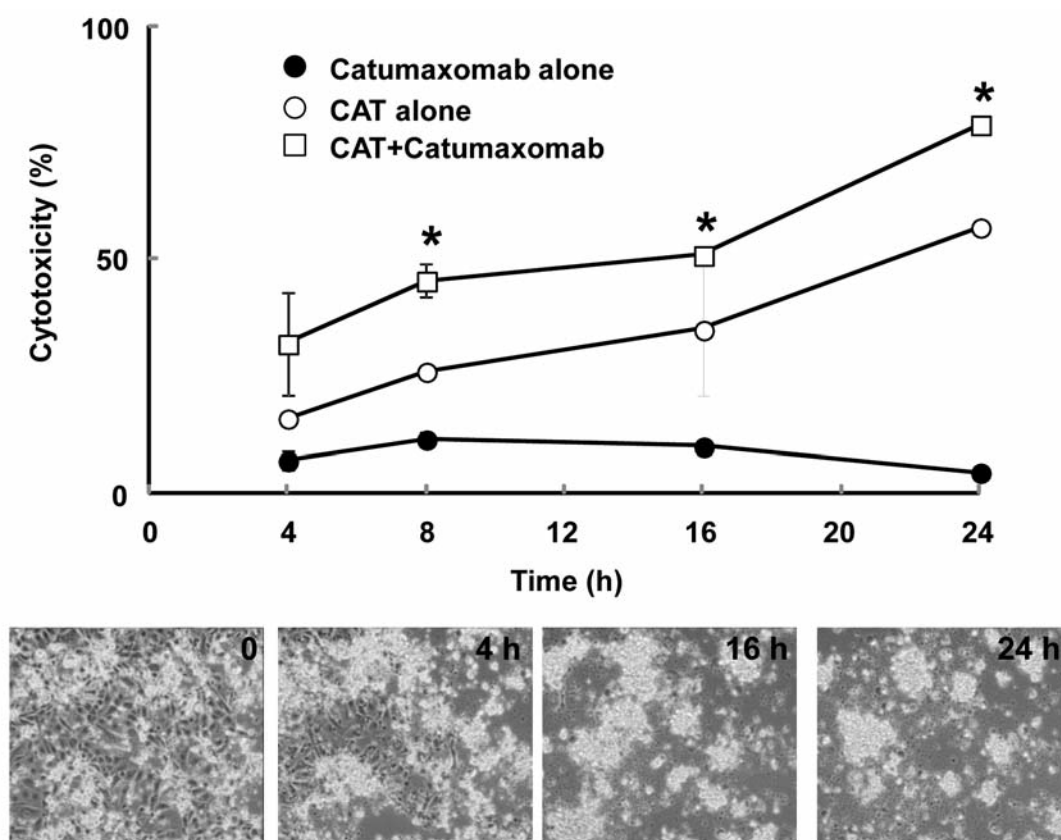


Figure 6. Time dependence of the catumaxomab-mediated cytotoxic activity of CATs against MU-PK1-hypoxia cells. Effects of catumaxomab-dependent activated T-cells on the viability of MU-PK1 cells. CATs were used at an effector:target ratio of 40:1. CATs were co-cultured with the CSLCs in the presence of different concentrations of catumaxomab for 4 h or a fixed dose of catumaxomab (10 ng/ml) for different culture times. An ATD cytotoxicity assay was then performed as described in the Materials and Methods. \* $p < 0.001$ .

associated with signalling activity (21-23). EpCAM is the best candidate for immunotherapy because it is widely and consistently expressed on CSLCs including those in pancreatic, liver, breast, and prostate cancer (11, 23-26). Therefore, we chose EpCAM as the molecular target for CSLCs. Studies have shown that antibodies against EpCAM are effective for immunotherapeutic approaches in pre-clinical and clinical trials (11, 12). In cancer immunotherapy, retargeting of T-cells to tumour cells by bi-specific T-cell-engaging antibodies is an appealing therapeutic concept because cytotoxic T-cells are among the most potent effector cells. The tri-functional antibody catumaxomab effectively attaches to both tumour and T-cells *via* its EpCAM- and CD3-specific binding sites, respectively, together with binding to Fc receptors (12). Because catumaxomab binds to both EpCAM on tumour cells and CD3 on T-cells, the combination of T-cell therapy with catumaxomab can evoke ADCC against EpCAM-expressing tumour cells. Clinical studies of antibodies to EpCAM are currently being undertaken (12). However, these

studies focus on the efficacy of the antibody alone. Our study demonstrates that activated T-cells together with catumaxomab have the strongest cytotoxicity against cancer cells, even after their progression to CSLCs. Thus, clinical studies are warranted for combinatorial therapy using cytotoxic T-cells with catumaxomab.

Indeed, adoptive T-cell immunotherapy using CATs is a promising cancer therapy, although the clinical effects have been modest using such therapies alone (15). For example, Dudley *et al.* demonstrated that the combination of non-myeloablative chemoradiation therapy with autologous activated tumour-infiltrating lymphocytes had clinical effects with durable tumour responses in 49-72% of patients (27, 28). In this study, we demonstrated that activated T-cells combined with catumaxomab are much more cytotoxic to CSLCs than are resting T-cells combined with catumaxomab. Thus, immunotherapeutic approaches with adoptive T-cell transfer plus a bi-specific antibody may be effective for treating drug-resistant tumours with stem cell-like properties.



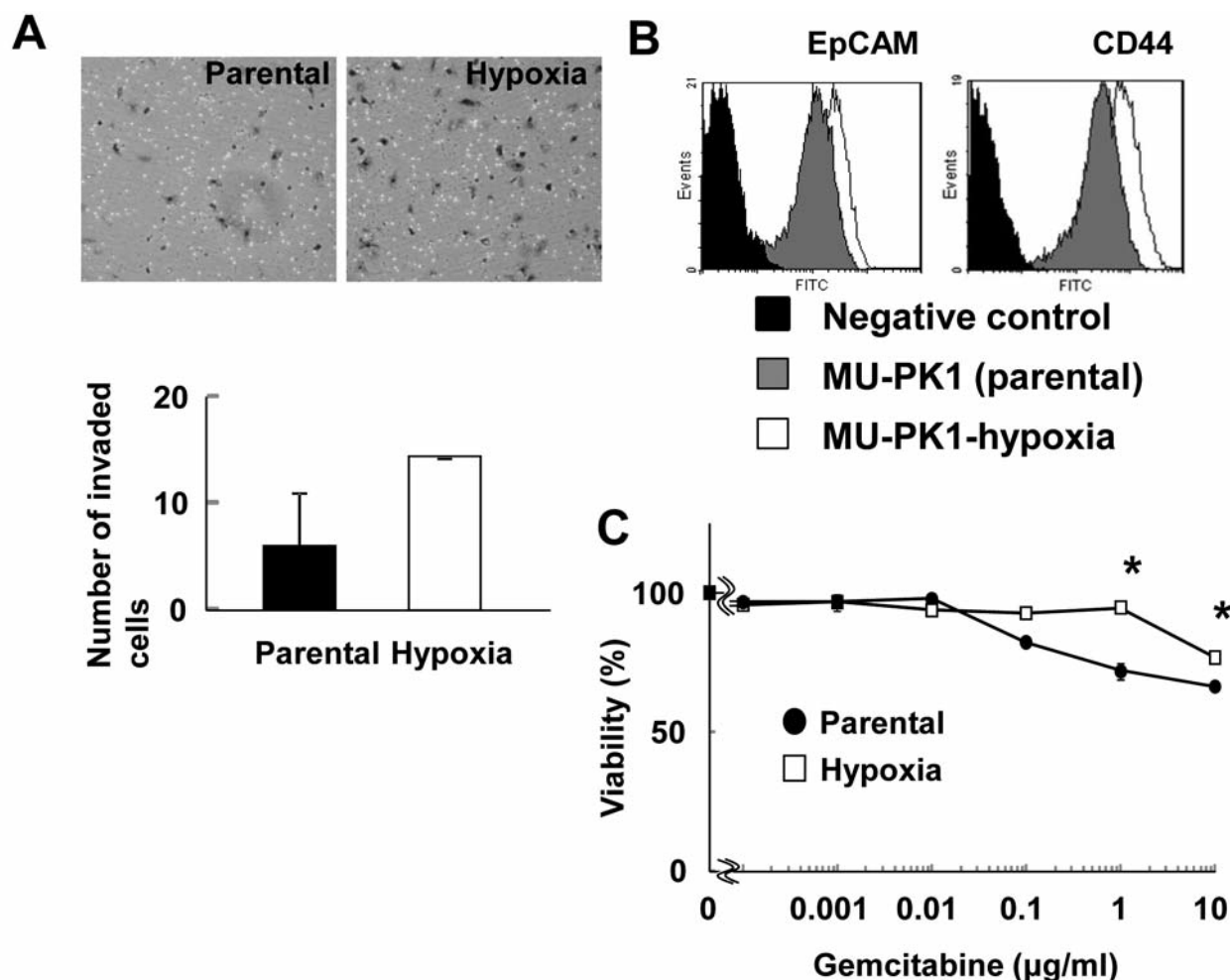


Figure 7. Characterization of cancer stem cell-like properties of parental and MU-PK1-hypoxia cells. **A**: Invasive capacity of parental and hypoxia-treated MU-PK1 cells. Representative inserts from invasion assays of parental and MU-PK1-hypoxia cells are shown. Membranes coated with Matrigel in cell culture inserts were used for invasion assays. For each insert, cells were counted in five fields. **B**: Flow cytometric analysis showing the relative expression of CD44 and EpCAM in both cell lines. MFIs of CD44 and EpCAM in each cell line are shown. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . **C**: Effect of gemcitabine on the growth of parental and MU-PK1-hypoxia cells. Parental and MU-PK1-hypoxia cells were treated with gemcitabine at different concentrations for 48 h. A WST-8 cell proliferation assay was then performed. For each concentration, five replicates were analyzed in a single experiment, and representative data from three experiments are shown. \* $p < 0.05$ .

Recent studies have shown that another EpCAM/CD3-bispecific antibody, MT110, combined with resting T-cells has cytotoxic activity against human colorectal tumour-initiating cells (12). The results were similar to our findings demonstrating that the combination of the EpCAM/CD3-bispecific antibody catumaxomab plus T-cells had a cytotoxic activity against CSLCs. However, the culture time required for cytotoxicity in the previous study was quite long at about 14 days. In contrast, we found that the incubation time required to eradicate CSLCs by combining catumaxomab with activated T-cells was 4-8 h. These results suggest that catumaxomab combined with activated T-cells, but not

resting T-cells, elicits cytotoxicity against CSLCs more effectively and in a shorter time period. Activated T-cells exhibit a greater cytotoxic effect on tumour cells than that of resting T-cells through up-regulation of NKG2D expression (29, 30). Because activated T-cells possess higher expression of NKG2D than do resting T-cells, catumaxomab-dependent cytotoxicity of activated T-cells appears to be associated with increased expression of costimulatory molecules.

A microenvironmental approach to isolate CSLCs is hypoxic culture (31, 32). Hypoxia induces hypoxia-inducible factor-1 $\alpha$  expression and then stem cell-like properties (31). In addition, hypoxia not only accounts for tissue necrosis but

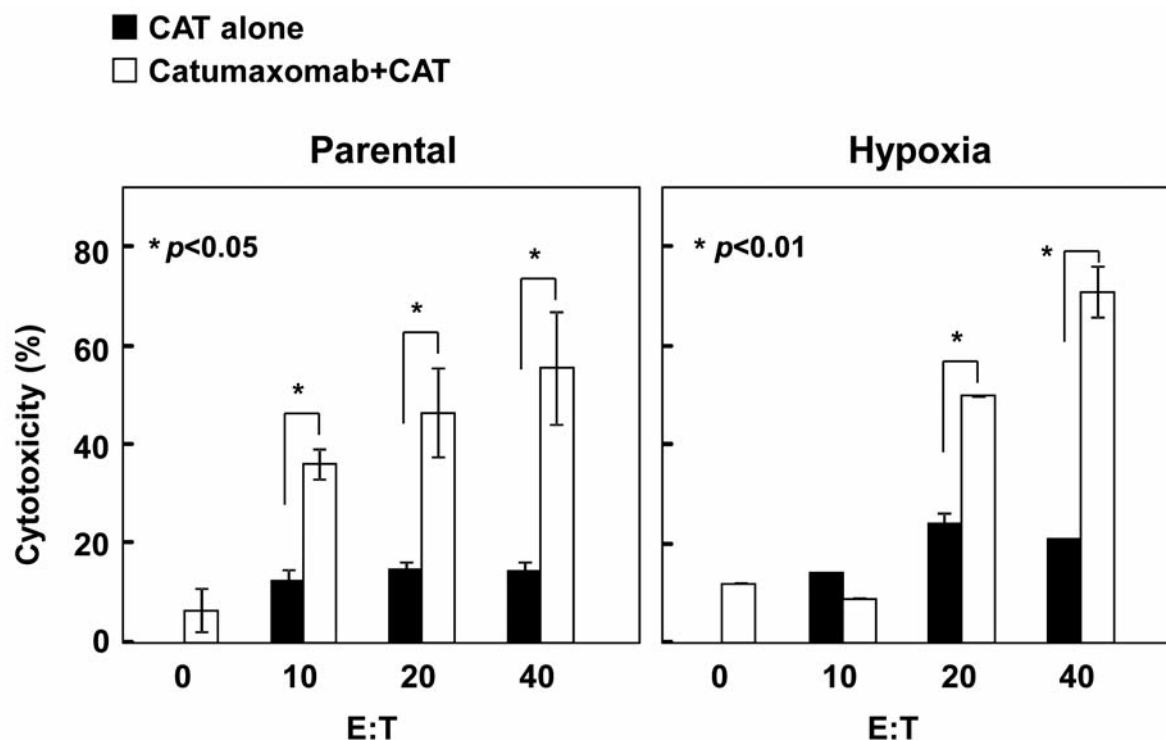


Figure 8. Comparison of cytotoxicity in parental and MU-PK1-hypoxia cells induced by catumaxomab plus CATs. Parental and MU-PK1-hypoxia cells were incubated with CATs at an effector to target cell ratio of 20:1, together with different concentrations of catumaxomab (0, 1, and 10 ng/ml) for 4 h. Data are representative of three independent experiments and are shown as mean values from triplicates within a single experiment. Error bars denote the standard deviation. Higher levels of ADCC induced by catumaxomab plus CD3-activated T-cells was evident against MU-PK1-hypoxia cells than those against parental cells. Note: The cytotoxicity of CATs-alone was also more evident against MU-PK1-hypoxia cells than that against parental cells. \* $p < 0.05$  (compared to CAT-alone).

also has a strong effect on tumour cell biology by reducing the sensitivity to apoptosis and other cell death signals and increasing signaling to promote angiogenesis, proliferation, and metastasis (31, 32). Furthermore, tumour hypoxia is a major problem for radiation therapy, and has been implicated in the development of resistance to many conventional chemotherapeutic agents (31-33). In the present study, we found that hypoxia induced more prominent stem cell-like properties in MU-PK1 cells. However, the combination of CATs plus catumaxomab lysed both MU-PK1-hypoxia cells and the parental cells at similar levels.

Collectively, these findings suggest the possibility of combinatorial therapy with catumaxomab and activated T-cells for treating chemoresistant pancreatic cancer. Currently, catumaxomab is used for immunotherapy of malignant ascites. Our findings may be useful for future combinatorial therapies using catumaxomab and adoptive T-cell immunotherapy.

In conclusion, our results warrant further clinical validation by combining catumaxomab and activated T-cell therapy for treating recurrent or drug-resistant tumours containing CSLCs.

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