Anti-glypican-1 antibody–drug conjugate is a potential therapy against pancreatic cancer

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BACKGROUND: Pancreatic cancer (PDAC) is the most lethal malignancy. New treatment options for it are urgently required. The aim was to develop an antibody–drug conjugate (ADC) targeting glypican-1 (GPC-1) as a new therapy for PDAC.

METHODS: We evaluated GPC-1 expression in resected PDAC specimens and PDAC cell lines. We then measured the antitumour effect of anti-GPC-1 monoclonal antibody conjugated with the cytotoxic agent monomethyl auristatin F (MMAF) in vitro and in vivo.

RESULTS: GPC-1 was overexpressed in most primary PDAC cells and tissues. The PDAC cell lines BxPC-3 and T3M-4 strongly expressed GPC-1 relative to SUIT-2 cells. Compared with control ADC, GPC-1-ADC showed a potent antitumour effect against BxPC-3 and T3M-4, but little activity against SUIT-2 cells. In the xenograft and patient-derived tumour models, GPC-1-ADC significantly and potently inhibited tumour growth in a dose-dependent manner. GPC-1-ADC-mediated G2/M-phase cell cycle arrest was detected in the tumour tissues of GPC-1-ADC-treated mice relative to those of control-ADC-treated mice.

CONCLUSIONS: GPC-1-ADC showed significant tumour growth inhibition against GPC-1-positive pancreatic cell lines and patient-derived, GPC-1-positive pancreatic cancer tissues. Our preclinical data demonstrated that targeting GPC-1 with ADC is a promising therapy for patients with GPC-1-positive pancreatic cancer.

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antigen for oesophageal squamous cell carcinoma (ESCC) using quantitative proteomics targeting the cell surface membrane protein. GPC-1 expression was very weak or undetectable in the heart, kidney, ovary, placenta, adrenal gland, thyroid, lung, liver, pancreas, stomach, small intestine, colon, prostate, thymus and brain.\(^\text{19,20}\) Thus, GPC-1 is a promising target for ESCC. It has recently been reported that GPC-1 was expressed in PDAC.\(^\text{21,22}\)

We produced a new ADC system using anti-GPC-1 monoclonal antibody and monomethyl auristatin F (MMAF), and demonstrated its potential effectiveness against uterine cervical cancer.\(^\text{23}\) The aims of this study were to investigate the GPC-1 expression in pancreatic cancer, and assess the feasibility of applying GPC-1-ADC as a new drug delivery technology.

**MATERIALS AND METHODS**

Patients and biopsy materials

Pancreatic cancer tissue was obtained from 75 patients who underwent RO pancreatectomy at the Department of Gastroenterological Surgery, Osaka University Hospital, between 2008 and 2012. Informed consent was obtained from all donors. All studies involving human subjects were approved by the Institutional Review Board (No. 15478-4) of Osaka University Hospital and by the National Institute of Biomedical Innovation, Health and Nutrition (No. 94). Diagnoses of all tumours as pancreatic cancer were confirmed following histological review by board-certified pathologists. TNM 7th edition (Union for International Cancer Control (UICC)) criteria were used to categorise pathological staging.

Immunohistochemistry

Three-micrometre sections were prepared from formalin-fixed, paraffin-embedded tissue samples. As described previously,\(^\text{19}\) the sections were deparaffinised with xylene and rehydrated in four graded alcohol solutions (70%, 80%, 90% and 100%). Immunohistochemical (IHC) staining for GPC-1 was performed using rabbit polyclonal anti-GPC-1 antibody (Atlas Antibodies AB, Stockholm, Sweden, 1:400) and visualised with Envision ChemMate (Dako, Glostrup, Denmark) according to the manufacturer’s protocol.

Immunostaining was scored according to the intensity of the staining: 0, no staining; 1, normal staining; 2, strong staining. The ‘density’ of staining (termed the positivity score) was as follows: 1, indicates less than 50% positivity; 2, indicates more than 50% positivity. The final IHC score was determined by multiplying the intensity score by the positivity score, resulting in a maximum possible score of 4. These data were referred to as the GPC-1 score. Furthermore, we divided patients into two equally balanced groups by score.

Cell lines and culture

BxPC-3 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). SUIT-2 cells were acquired from the Japanese Collection of Research Bioresources (Osaka, Japan). T3M-4 cells were procured from the RIKEN BioResource Center (Wako, Japan). BxPC-3 and SUIT-2 cells were maintained in RPMI 1640 medium. T3M-4 cells were maintained in Ham’s F-10 medium. All media were supplemented with 10% foetal bovine serum (FBS; Serum Source International, Charlotte, NC, USA) and 100 U mL\(^{-1}\) penicillin + 100 \(\mu\)g mL\(^{-1}\) streptomycin (Nacalai Tesque Inc., Kyoto, Japan). Cultures were maintained at 37 °C under a humidified atmosphere at 5% CO\(_2\).

Antibody generation

To generate monoclonal antibodies (mAbs) against human GPC-1, 4–6-week mice with MRL or C3H backgrounds were immunised with recombinant human GPC-1 protein (R&D Systems, Minneapolis, MN, USA) as previously reported.\(^\text{23}\)

Preparation of antibody–drug conjugate

As type control antibody (mouse IgG2a, clone MOPC-173, Biologic, San Diego, CA, USA) were used to synthesise the ADC. The GPC-1 mAb was partially reduced with tris-(2-carboxyethylphosphine) hydrochloride (TCEP) followed by reaction with maleimidolcaproyl–valine–citrulline–p-aminobenzyloxycarbonyl–monomethyl auristatin F (mc–vc–PABC–MMAF)\(^\text{24}\) to yield GPC-1-ADC and control ADC, respectively. To remove residual unreactive toxins, the conjugated ADCs were desalted on Sephadex G50 columns, and the buffer was replaced with phosphate-buffered saline (PBS) and filtered. The drug-to-antibody ratio (DAR) was determined from the ratio of A248 nm: A280 nm. The DAR was 4.1 for GPC-1-ADC and 3.8 for control ADC. The drug distribution was analysed by hydrophobic interaction chromatography (HIC).

Quantitative flow cytometric analysis

Pancreatic cancer cells were grown to 80% confluency in 100-mm dishes. As described previously,\(^\text{26}\) cells were washed twice in PBS (Nacalai Tesque Inc., Kyoto, Japan) and detached in 0.02% (w/v) EDTA (Nacalai Tesque Inc., Kyoto, Japan). Cells were washed twice with cold FACs buffer (PBS supplemented with 1% (w/v) FBS and 0.1% (w/v) sodium azide), then incubated with mouse anti-GPC-1 antibody (clone 01a033) at 10 \(\mu\)g mL\(^{-1}\) and labelled with FITC-labelled goat anti-mouse IgG (H + L chain-specific) antibody (Southern Biotech, Birmingham, AL, USA). Stained cells were viewed under a FACs Canto II cytometer (Becton Dickinson, Mountain View, CA, USA) and analysed with FlowJo software (Tree Star, Stanford, CA, USA).

As described previously,\(^\text{23}\) plasma membrane GPC-1 expression levels were quantified by QIFIKIT flow cytometric indirect immunofluorescence assay (Dako, Hamburg, Germany) using anti-GPC-1 mAb (clone 01a033) as the primary antibody. Per sample, 10\(^4\) cells were incubated in a saturating concentration (10 \(\mu\)g mL\(^{-1}\)) of primary antibody for 30 min at 4 °C. After washing, FITC-conjugated secondary antibody (1:50) was added for 45 min at 4 °C. Antibody binding was measured in a FACs Canto II cytometer (Becton Dickinson, Mountain View, CA, USA). The specific antigen density was calculated by subtracting the background antibody equivalent from the antibody-binding capacity based on a standard curve of log mean fluorescence intensity against log antibody-binding capacity.

Cell proliferation assay

Pancreatic cancer cells were plated in 96-well plates at a density of 1500 cells well\(^{-1}\) (90 \(\mu\)L well\(^{-1}\)) and incubated at 37 °C under a 5% CO\(_2\) atmosphere. After overnight incubation, various concentrations of MMAF (0–1000 nM), anti-GPC-1 antibody 01a033 (0–16 nM), GPC-1-ADC (0–16 nM) and control ADC (0–16 nM) were added. Cell viability was assessed after 144 h using a Cell Titer-Glo luminescent assay kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Percentage survival was calculated by dividing the measured luminescence per drug or ADC concentration by the mean number of untreated cells (in growth medium), and multiplying the quotient by 100. The IC\(_{50}\) was then calculated.

Internalisation studies

BxPC-3 and T3M-4 cells (10\(^4\) tube\(^{-1}\)) were incubated for 1 h at 4 °C on ice, and then incubated for 30 min with 16 nM GPC-1-ADC (clone 01a033). The cells were then partitioned into two groups. For one group, internalisation was assessed upon incubation at 37 °C (100 \(\mu\)L vial\(^{-1}\)). The other group served as a control for total cell surface binding, and was incubated at 4 °C. After the indicated incubation times, the cells were washed thrice with ice-cold PBS–0.2% (w/v) BSA buffer. The cells were incubated with 1 \(\mu\)g mL\(^{-1}\) biotinylated mouse anti-GPC-1 antibody (clone 02b006) for 30 min at 4 °C. The remaining surface expression was...
In vivo efficacy study in patient-derived xenograft model
The use of human tissues was permitted by the Ethics Committees of the Osaka University Graduate School of Medicine and the National Institute of Biomedical Innovation, Health and Nutrition. Surgically resected samples from a patient who received no preoperative radiation or chemotherapy were cut into 3–4-mm pieces and subcutaneously transplanted into 6-week female NOD/ Shi-scid-IL2R null (NOG) mice. Mice were housed in a temperature-controlled room with a 12-h light/12-h dark cycle and provided free access to water. Mice were observed daily for tumour growth. The tumours were measured once or twice. Mice were anaesthetised by 3% isoflurane and euthanised via cervical dislocation 36 days after the first treatment. Then, tumours were resected and weighed.

To investigate the pharmacologic action of GPC-1-ADC at the cellular level, animals with the BxPC-3 tumour xenograft were injected with PBS, control ADC (10 mg kg\(^{-1}\)) or GPC-1-ADC (1 mg kg\(^{-1}\), 3 mg kg\(^{-1}\) or 10 mg kg\(^{-1}\)), and the tumours were harvested 24 h later. Tumours were fixed in formalin, embedded in paraffin and sliced into 3-μm sections. IHC was performed using anti-phospho-histone H3 (Ser10) (#9701, 1:400, Cell Signaling Technologies, Danvers, MA, USA). In vivo efficacy study in pancreatic cancer cell line xenograft model

Establishment of GPC-1-knockdown cells
To generate a stable GPC-1-knockdown cell line, BxPC-3 cells were transfected with a commercial plasmid vector expressing sh-mRNA (shRNA) targeting GPC-1 mRNA or a negative nonspecific shRNA control (SuperArray Bioscience Corp., Frederick, MD, USA) using LipoFectamineDM 2000 (Life Technologies Corp., Carlsbad, CA, USA). Transfected cells were selected using 600 μg mL\(^{-1}\) G418 (Life Technologies Corp., Carlsbad, CA, USA) and maintained in 250 μg mL\(^{-1}\) G418. A GPC-1-knockdown cell line was established and designated BxPC-3 KD-2-3. A BxPC-3 control cell line was established and designated BxPC-3 NC-11 via stable transfection with an empty vector. GPC-1 knockdown of transfected cells was assessed by fluorescence-activated cell sorting (FACS) and quantitative reverse transcription-PCR (qRT-PCR).

Quantitative reverse transcription-PCR (qRT-PCR) analysis
BxPC-3, BxPC-3 NC-11 and BxPC-3 KD-2-3 cells were cultured in six-well plates at a density of 4.0 × 10\(^4\) cells well\(^{-1}\). After 24 h, total RNA was extracted and purified using RNeasy Mini Kit (QIAGEN, Valencia, CA), and cDNA was prepared using Quantitect Reverse Transcription Kits (QIAGEN). To confirm the expression of GPC-1, qRT-PCR was performed as previously described.23 β-actin was used as a housekeeping gene for quantitative real-time PCR normalisation. Primer sequences were used as follows: GPC-1, forward primer 5′-GCCAGATCTAGGCAAGCGAG-3′ and reverse primer 5′-AGGTCTCTCCATCTGCT-3′ and β-actin, forward primer 5′-GGGACGCCCAACACGACA-3′ and reverse primer 5′-CTCTTTATGTCCGACCGATTTC-3′.

In vivo efficacy study in pancreatic cancer cell line xenograft model
In this study, since it is essential to provide the efficacy and safety of this drug in animal models, we carried out animal experiments basically based on the previous report.22 Healthy female CB17/severe combined immunodeficient (SCID) mice aged 6 weeks were obtained from Charles River Japan (Yokohama, Japan). The animals were maintained in a pathogen-free facility in the National Institute of Biomedical Innovation, Health and Nutrition. Mice were housed in a temperature-controlled room with a 12-h light/12-h dark cycle, and provided free access to water. For the xenograft experiments, the mice were anaesthetised by 3% isoflurane, and subcutaneously injected in the flank with 5 × 10\(^6\) BxPC-3 cells in 100 μL of 1:1 (v/v) PBS:Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). When the tumour volumes were >100 mm\(^3\), the mice were randomly divided into five groups (8–9 per group). PBS, control ADC (10 mg kg\(^{-1}\)) or GPC-1-ADC (1 mg kg\(^{-1}\), 3 mg kg\(^{-1}\) or 10 mg kg\(^{-1}\)) was injected from caudal veins every 4 days until four doses had been administered. Tumour sizes were measured every 4 days using a vernier calliper. Tumour volumes were calculated as W\(^2\) × L/2, where W = width, L = length and V = volume. Body weights of mice were measured every 4 days. Mice were anaesthetised by 3% isoflurane and euthanised via cervical dislocation 36 days after the first treatment. Then, tumours were resected and weighed.

RESULTS
GPC-1 expression in pancreatic cancer specimens
Among the 75 cases, we divided them into 2 equally balanced groups by GPC-1-stained score. Thirty-three cases (44%) scored...
more than 2 points (denoted as our high-expression group (HG), Fig. 1a, left panel). The remaining 42 cases (56%) scored less than 1 point (low-expression group (LG), Fig. 1a, right panel). The distribution of GPC-1 expression is shown in Fig. 1b.

We analysed the relation between GPC-1 expression in pancreatic cancer and various clinicopathological parameters, and found the correlation between high expression of GPC-1 and lymph node metastasis (Supplementary Table 1). Furthermore, GPC-1 expression significantly correlated with poor OS ($P = 0.004$, log-rank test) (Fig. 1c) and RFS ($P = 0.021$, log-rank test) (Fig. 1d).

GPC-1 expression in human pancreatic cancer cell lines

Flow cytometry was performed to measure GPC-1 protein on pancreatic cancer cell surfaces. GPC-1 expression was high in BxPC-3 and T3M-4 and low in SUIT-2 (Fig. 2a). Quantitation of GPC-1 expression on the plasma membrane by indirect immunofluorescence assay indicated that it was upregulated in BxPC-3 (93290 sites cell$^{-1}$) and T3M-4 (76850 sites cell$^{-1}$) but expressed at low levels in SUIT-2 (25444 sites cell$^{-1}$) (Table 1).

Cytotoxicity studies with GPC-1-ADC

A cell growth assay with ADCs was performed using the GPC-1-positive pancreatic cancer cell lines BxPC-3 and T3M-4. SUIT-2 served as a negative control. Unconjugated anti-GPC-1 mAb had no effect on the viability of any cell line (Fig. 2b). Nevertheless, GPC-1-ADC caused a dose-dependent decrease in the viability of BxPC-3 and T3M-4 in vitro (Fig. 2c). The IC$_{50}$ values of GPC-1-ADC were 0.063 nM for BxPC-3 and 0.24 nM for T3M-4, respectively. However, GPC-1-ADC had little effect on SUIT-2 cells (Fig. 2c). The IC$_{50}$ of GPC-1-ADC was not calculated for SUIT-2 as the 16-nM cell inhibitory rate of GPC-1-ADC did not reach 50% (Fig. 2c). Unconjugated GPC-1 mAb was not cytotoxic at concentrations < 666.6 nM (data not shown). As MMAF impairs plasma membrane permeability, MMAF sensitivity was low. The IC$_{50}$ values for MMAF against the cell lines were in the range of 24.4–459.5 nM (Table 1).

Internalisation of GPC-1-ADC

The binding capacity and percentage of internalisation of GPC-1-ADC were determined for BxPC-3 and T3M-4 by flow cytometry. Residual cell surface GPC-1 was measured after each GPC-1-ADC exposure time point using biotinylated anti-GPC-1 mAb (clone 02b006). GPC-1-ADC internalisation occurred rapidly in both cell lines (Fig. 2d). An immunofluorescence analysis was conducted to confirm GPC-1-ADC translocation to the lysosomes. GPC-1-ADC bound to the membranes of cells preincubated at 4°C. When BxPC-3 exposed to GPC-1-ADC was incubated at 37°C for 2 h, GPC-1-ADC appeared in the lysosomes. It overlapped with the lysosomal marker LAMP-1 (Fig. 2e). Thus, GPC-1-ADC first binds to the GPC-1 on the membranes of GPC-1-expressing cells, is internalised and then translocates to the lysosomes.

Cytotoxicity studies with GPC-1-knockdown cell line

We investigated the association between GPC-1 expression and GPC-1-ADC cytotoxicity using GPC-1-knockdown BxPC-3. Both BxPC-3 and BxPC-3 NC-11 (negative control cell line) expressed GPC-1, whereas the GPC-1-knockdown BxPC-3 KD-2-23 was GPC-1-negative according to flow cytometry (Fig. 3a) and qRT-PCR (Fig. 3b). We performed a cell growth assay, and confirmed that GPC-1-ADC reduced BxPC-3 and BxPC-3 NC-11 viability in a dose-dependent manner. In contrast, GPC-1-ADC had little effect on BxPC-3 KD-2-23 (Fig. 3c, Supplementary Table 2).

In vivo efficacy study in BxPC-3 xenograft

SCID mice were subcutaneously inoculated with BxPC-3 cells, and then intravenously treated with 1 mg kg$^{-1}$, 3 mg kg$^{-1}$ or 10 mg kg$^{-1}$ GPC-1-ADC once every 4 days for a total of four doses (Fig. 4a). GPC-1 expression in the BxPC-3 xenograft was confirmed by immunohistochemistry (IHC) (Fig. 4a). Compared with PBS and control ADC, GPC-1-ADC administration significantly inhibited BxPC-3 xenograft growth as assessed by tumour volume and weight (Fig. 4a, b). Tumour volume was significantly decreased by 10 mg kg$^{-1}$ GPC-1-ADC. No significant weight loss was observed in
any group (Fig. 4c). The BxPC-3 xenograft tumours were stained with phosphorylated histone H3 (Ser10), which is related to chromosome condensation. The aim was to analyse the pharmacologic action of GPC-1-ADC in vivo using a mitotic marker antibody. A dramatic increase in the percentage of mitotically active tumour cells was observed following GPC-1-ADC treatment but not in the control ADC (Fig. 4d, e). Therefore, the tubulin-polymerising inhibitor MMAF was successfully delivered to the
GPC-1 monoclonal antibody. Furthermore, the GPC-1-knockdown cell (KD-2–23) derived from GPC-1-positive BxPC-3 was also relatively insensitive to GPC-1-ADC, whilst those expressing GPC-1 only at low levels (such as SUIT-2) were comparatively resistant to it. GPC-1-positive and GPC-1-overexpression was related to poor prognosis. We demonstrated that targeting GPC-1 with anti-GPC-1 mAb (clone 1–12) had a strong antitumour effect via ADCC and CDC in a GPC-1-positive ESCC xenograft model.19,20 We showed the efficacy of anti-GPC-1 mAb after confirming GPC-1 expression in PDAC. However, we could not prove that it had the same efficacy as ESCC (Supplementary Fig. 1). GPC-1 expression may be weaker in pancreatic than oesophageal cancer, and its roles may differ in each case.

We reported the establishment of a new ADC-based system combining anti-GPC-1 mAb with MMAF. The latter inhibits mitosis by suppressing tubulin polymerisation and disrupting the microtubule network in proliferating cells. It has shown remarkable efficacy against uterine cervical cancer.23 ADC efficacy depends mainly on target antigen expression on tumour cells, ADC-binding affinity to the antigen, cellular internalisation and conjugated drug potency. Our in vitro ADC assay showed that cancer cells highly expressing GPC-1 (namely BxPC-3 and T3M-4) were highly sensitive to GPC-1-ADC, whilst those expressing GPC-1 only at low levels (such as SUIT-2) were comparatively resistant to it. Furthermore, the GPC-1-knockdown cell (KD-2–23) derived from GPC-1-positive BxPC-3 was also relatively insensitive to GPC-1-ADC. Thus, GPC-1 expression is necessary to enable GPC-1-ADC to inhibit cancer cell growth. Moreover, anti-GPC-1 mAb was rapidly internalised into the cancer cells after GPC-1 bound to them. Therefore, GPC-1 is a suitable target for ADC. GPC-1 expression was very weak or undetectable in various normal cells.20 For this reason, specific efficacy of GPC-1-ADC to PDAC is expected. Since there are some reports that GPC-1 is reported to be shed into the extracellular matrix, and high GPC-1-expression tumours secrete a lot of GPC-1 and lead to high serum GPC-1 level,21,26 we might predict an effect of GPC-1-ADC by measuring the serum GPC-1 levels.

To formulate GPC-1-ADC, we conjugated MMAF with anti-GPC-1 mAb. MMAF inhibits mitosis by suppressing tubulin polymerisation and disrupting the microtubule network in proliferating cells. MMAF is a novel auristatin derivative with a charged N-terminal phenylalanine that attenuates its cytotoxicity compared with its uncharged counterpart monomethyl auristatin E (MMAE).24,27–29 The carboxylic acid terminus of free MMAF limits its passive transit through cell membranes.29 MMAF has potent antitumour GPC-1-expressing tumour cells via anti-GPC-1 mAb and caused mitotic arrest.

In vivo efficacy study in patient-derived xenograft We also assessed the antitumour efficacy of GPC-1-ADC against a pancreatic cancer patient tumour-derived xenograft (PDX). Pancreatic cancer tissues were subcutaneously implanted in NOD mice. The animals then received intravenous 1 mg kg−1, 3 mg kg−1 or 10 mg kg−1 GPC-1-ADC every 4 days for a total of four doses (Fig. 5a). GPC-1 expression in the tumour tissue was confirmed by IHC (Fig. 5a). PDX tumour growth in the 10 mg kg−1 GPC-1-ADC group was significantly suppressed relative to the control-ADC group (Fig. 5a, b). No significant weight loss was observed in any group (Fig. 5c). The PDX tumours were stained with phosphorylated histone H3 (Ser10). A substantial increase in the percentage of mitotic tumour cells was detected following GPC-1-ADC treatment but not in response to the control ADC (Fig. 5d, e).

**DISCUSSION**

We previously identified glypican-1 (GPC-1) by quantitative proteomics as an antigen for oesophageal squamous cell carcinoma (ESCC). We focused on the cell membrane protein and confirmed relatively low GPC-1 expression in normal tissues relative to ESCC.19 GPC-1 expression in ESCC is related to poor prognosis and chemoresistance, as previously reported.19 In this study, IHC analyses showed that 97.3% of PDAC specimens were GPC-1 positive, and GPC-1 overexpression was related to poor prognosis. We demonstrated that targeting GPC-1 with anti-GPC-1 mAb had a strong antitumour effect via ADCC and CDC in a GPC-1-positive ESCC xenograft model.19,20 We showed the efficacy of anti-GPC-1 mAb after confirming GPC-1 expression in PDAC. However, we could not prove that it had the same efficacy as ESCC (Supplementary Fig. 1). GPC-1 expression may be weaker in pancreatic than oesophageal cancer, and its roles may differ in each case.

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Anti-glypican-1 antibody-drug conjugate is a potential therapy for pancreatic ductal adenocarcinoma (PDAC).

GPC-1-ADC had a significant antitumour effect in BxPC-3 xenografted mice. Thence, it activates the antimitotic mechanism.29,30 Furthermore, the extent to which ADC mediates bystander killing depends largely on ADC internalisation after it binds to the target antigen, whether the linker is cleavable, and the hydrophobicity of the cytotoxic warhead. All of these factors must be established for the clinical applications.

There were several limitations to this study. First, GPC-1 expression was heterogeneous in clinical pancreatic cancer. This discrepancy may account for the observed differences in GPC-1-ADC antitumour efficacy among the pancreatic cancer cell lines. Nevertheless, recent studies indicated that the chemistry of the drug may determine whether it can diffuse into the surrounding cells and cause ‘bystander killing’.29,30 Furthermore, the extent to which ADC mediates bystander killing depends largely on ADC internalisation after it binds to the target antigen, whether the linker is cleavable, and the hydrophobicity of the cytotoxic warhead. All of these factors must be established for the clinical applications.
application of GPC-1-ADC. Second, we must develop GPC-1-ADC using humanised anti-GPC-1 monoclonal antibody, and validate its clinical efficacy and safety. We selected an anti-GPC-1 antibody with strong internalisation capacity.\textsuperscript{23} GPC-1-ADC immediately internalised GPC-1-positive cancer cells. We may be able to develop more efficacious GPC-1-ADC using antibodies with high internalisation activity.

The GPC-1-ADC developed in this study showed significant tumour growth inhibition against GPC-1-positive pancreatic cancer cells and patient-derived, GPC-1-positive pancreatic cancers. Our preclinical data demonstrated that targeting GPC-1 by ADC is a promising therapy for patients with GPC-1-positive pancreatic cancer.

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**AUTHOR CONTRIBUTIONS**

Conception and design: T. Nishigaki, T.T. and T. Naka; methodology development: T. Nishigaki, T.T., H.H., S.S., M.F., T.O. and T. Naka; data acquisition: T. Nishigaki, T.O., Y.S., S.T. and S.S.; human pancreatic cancer tissue: H.E.; patient-derived xenograft: T. Nomura; data analysis and interpretation: T. Nishigaki, T.T., H.H., S.S., K.T., Y.M., T.M., Y.K., K.N., M.M. and Y.D. and T. Naka; paper writing, review and/or revision: T. Nishigaki, T.T., S.S., M.M. and Y.D.; administrative, technical or material support: S.S.; study supervision: T. Naka.

**ADDITIONAL INFORMATION**

**Ethics approval and consent to participate** This study was performed in accordance with the Declaration of Helsinki. All studies involving human subjects were approved by the Institutional Review Board (No. 15478-4) of Osaka University Hospital and by the National Institute of Biomedical Innovation, Health and Nutrition (No. 94). Written informed consent was obtained from all participants. All animal experiments were approved by the Institutional Review Board of the National Institute of Biomedical Innovation, Health and Nutrition (No. D525-39R1).

**Consent to publish** Not applicable.

**Data availability** All data generated or analysed during this study are included in this published article and its supplementary information files.

**Competing interests** The authors declare no competing interests.

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