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Antipodocalyxin Antibody chPcMab-47 Exerts Antitumor Activity in Mouse Xenograft Models of Colorectal Adenocarcinomas

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Podocalyxin (PODXL) is expressed in several cancers, including brain tumors and colorectal cancers. PODXL overexpression is an independent predictor of progression, metastasis, and poor outcome. We recently immunized mice with recombinant human PODXL, which was produced using LN229 glioblastoma cells, and produced a clone PcMab-47 that could be used for investigating PODXL expression by flow cytometry and immunohistochemical analysis. Herein, we produced a human–mouse chimeric PcMab-47 (chPcMab-47) and investigated its antitumor activity against PODXL-expressing tumors. chPcMab-47 reacted with LN229, LN229/PODXL, and Chinese hamster ovary (CHO)/PODXL cells, but it did not react with CHO-K1 or PODXL-knockout LN229 cell line (PDIS-13). chPcMab-47 exerted antitumor activity against a mouse xenograft model using CHO/PODXL. Furthermore, chPcMab-47 was reactive with colorectal cancer cell lines such as HCT-15, Caco-2, HCT-8, and DLD-1. chPcMab-47 also exhibited antitumor activity against a mouse xenograft model using HCT-15. These results suggest that chPcMab-47 could be useful for antibody therapy against PODXL-expressing cancers.

Keywords: podocalyxin, PODXL, chimeric antibody

Introduction

Podocalyxin (PODXL) is a type I transmembrane protein with a molecular weight of 150,000–200,000, which was first found in podocytes of the rat kidney,[1–4] and its homologs have been reported in humans.[5,6] PODXL is a highly glycosylated sialomucin with *N*-glycan, *O*-glycan, and keratan sulfate[7] and is similar to CD34, which is known as a hematopoietic stem cell marker.[8]

PODXL is expressed in the kidney, heart, pancreas, and breast tissues and plays an important role in the development of several tissues.[9] PODXL acts as an adhesive molecule to bind to platelets or vascular endothelial cells[10,11]; however, it also functions as an antiadhesive molecule through its negatively charged mucin domain, including sialic acid and keratan sulfate, and for the formation and maintenance of the filtration slits between the podocyte processes in the kidney.[12] Furthermore, PODXL regulates cell morphology by associating with actin cytoskeletal proteins such as Na^+/H^+ exchanger regulatory factor-1/2 and ezrin.[13,14]

PODXL is known as one of the pluripotent stem cell markers. The antigen of monoclonal antibodies (mAbs) such as TRA-1-60 and TRA-1-81 is keratan sulfate, which is attached to PODXL.[4] PODXL is also a diagnostic marker and a prognostic indicator in several cancers, including brain tumors[7,15] and colorectal cancers,[16–19] indicating that the overexpression of PODXL in cancer is a potential target for antibody therapy. Recently, we established the anti-PODXL mAb, PcMab-47, for use in flow cytometry and immunohistochemistry.[20] In this study, we produced a human–

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mouse chimeric PcMab-47 (chPcMab-47) and investigated its antitumor activity against PODXL-expressing tumors.

Materials and Methods

Cell lines

LN229, Caco-2, HCT-8, and Chinese hamster ovary (CHO)-K1 cell lines were obtained from the American Type Culture Collection (Manassas, VA). HCT-15 and DLD-1 cell lines were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Miyagi, Japan). CHO-S cell line was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). LN229 and CHO-K1 cells were transfected with PODXL plasmids, which included the full length of PODXL, using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. LN229/PODXL-knockout (KO) cells (PDIS-13) were produced using CRISPR/Cas9 plasmids (Target ID: HS0000056763) against human PODXL (Sigma-Aldrich Corp., St. Louis, MO).[21] PDIS-13 is available from the Cell Bank of Kato's Lab (www.med-tohoku-antibody.com/topics/001_paper_cell.htm) in Tohoku University (Miyagi, Japan).

CHO-K1 and CHO-K1/PODXL cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing L-glutamine (Nacalai Tesque, Inc., Kyoto, Japan). LN229, LN229/PODXL, PDIS-13, Caco-2, HCT-8, HCT-15, and DLD-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (Nacalai Tesque, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. G418 (0.5 mg/mL; Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to the CHO-K1/PODXL and LN229/PODXL cultures. Antibiotics, including 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 25 μg/mL of amphotericin B (Nacalai Tesque, Inc.), were added to all media.

Antibodies

PcMab-47, a mouse anti-PODXL mAb (IgG_1, kappa), was developed as previously described.[21] Human IgG was purchased from Sigma-Aldrich Corp.. To generate the human–mouse chimeric anti-PODXL (chPcMab-47), appropriate V_H and V_L cDNAs of mouse PcMab-47 and C_H and C_L of human IgG_1 were subcloned into pCAG-Ble and pCAG-Neo vectors (Wako Pure Chemical Industries, Ltd.), respectively. Antibody expression vectors were transfected into CHO-S cells using the Lipofectamine LTX reagent (Thermo Fisher Scientific, Inc.). Stable transfectants of CHO-S/chPcMab-47 cells were selected by cultivating the transfectants in a medium containing 0.5 mg/mL of both geneticin and zeocin (InvivoGen, San Diego, CA). CHO-S/chPcMab-47 cells were cultivated in CHO-S-SFM II medium (Thermo Fisher Scientific, Inc.). chPcMab-47 was purified using Protein G-Sepharose (GE Healthcare Bio-Sciences, Pittsburgh, PA).

Flow cytometry

Cell lines were harvested by brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin in phosphate-buffered saline (PBS), cells were treated with primary mAbs for 30 minutes at 4°C, followed by treatment with fluorescein isothiocyanate (FITC)-labeled goat antihuman IgG (Thermo Fisher Scientific, Inc.). Fluorescence data were collected using the Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

Determination of the binding affinity using flow cytometry

LN229/PODXL (2×10^5 cells) cells were resuspended in 100 μL of serially diluted PcMab-47 or chPcMab-47 (0.6–10 μg/mL), followed by the addition of secondary antimouse IgG or antihuman IgG (Thermo Fisher Scientific, Inc.). Fluorescence data were collected using a cell analyzer

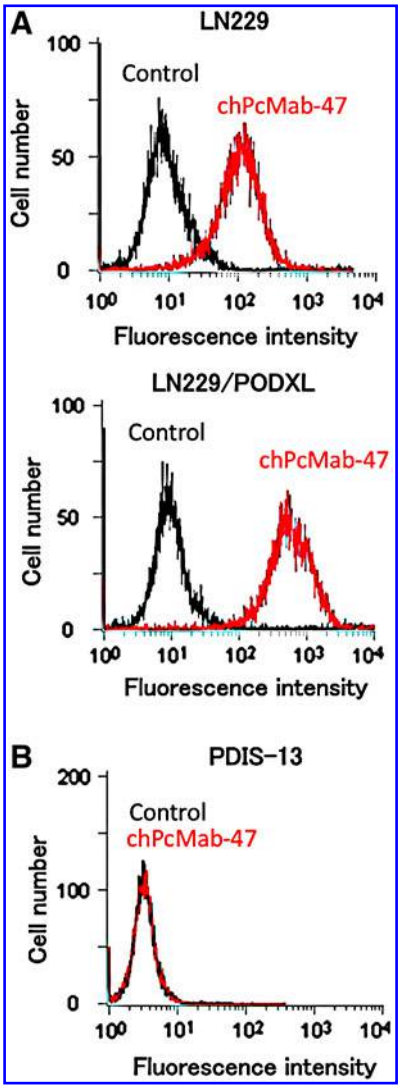

FIG. 1. Specific detection of PODXL by chPcMab-47 using flow cytometry. **(A)** LN229 and LN229/PODXL cells were treated with chPcMab-47 (1 μg/mL; red) or control PBS (black) for 30 minutes at 4°C, followed by treatment with FITC-labeled antihuman IgG. **(B)** PDIS-13 (LN229/PODXL-knockout cells) was treated with chPcMab-47 (1 μg/mL; red) or control PBS (black) for 30 minutes at 4°C, followed by treatment with FITC-labeled antihuman IgG. PBS, phosphate-buffered saline; PODXL, podocalyxin.

(EC800; Sony Corp.). The dissociation constants (K_D) were obtained by fitting the binding isotherms using the built-in one-site binding models in GraphPad PRISM 6 (GraphPad software, Inc., La Jolla, CA).

Antitumor activity of anti-PODXL antibodies

CHO/PODXL cells and HCT-15 cells were trypsinized and washed with PBS. The cell density was adjusted using PBS to 5.0×10^7 cells/mL, and 100 μL per animal of the cell suspension was subcutaneously inoculated into BALB/c nude mice. After 1 day, 100 μL of 1 mg/mL of chPcMab-47 and human IgG was injected into the peritoneal cavity of mice. Additional antibodies were injected once a week for several weeks. Human natural killer (NK) cells (8.0×10^5 cells; Takara Bio, Inc., Shiga, Japan) were injected around the tumors several times. The tumor diameter was measured every 3–7 days and was calculated using the following formula: volume = $W^2 \times L/2$, where W is the short diameter and L is the long diameter. The mice were euthanized 30 (CHO/PODXL) or 29 days (HCT-15) after cell implantation.

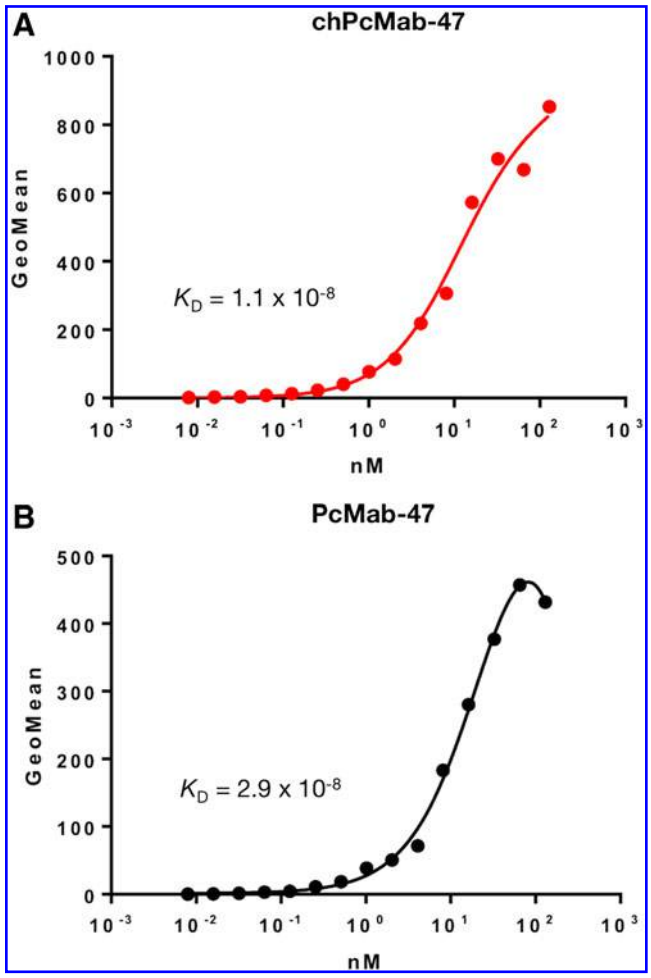

FIG. 2. Determination of the binding affinity of chPcMab-47 and PcMab-47 using flow cytometry. LN229/PODXL cells (2×10^5 cells) were resuspended in 100 μL of serially diluted chPcMab-47 **(A)** or PcMab-47 **(B)** (0.6–10 μg/mL), followed by addition of secondary antihuman IgG or antimouse IgG. Fluorescence data were collected using a cell analyzer.

Statistical analyses

All data were expressed as mean ± standard error of the mean. Two-way analysis of variance was performed as appropriate. p values <0.05 were considered to be statistically significant. All statistical tests were two sided.

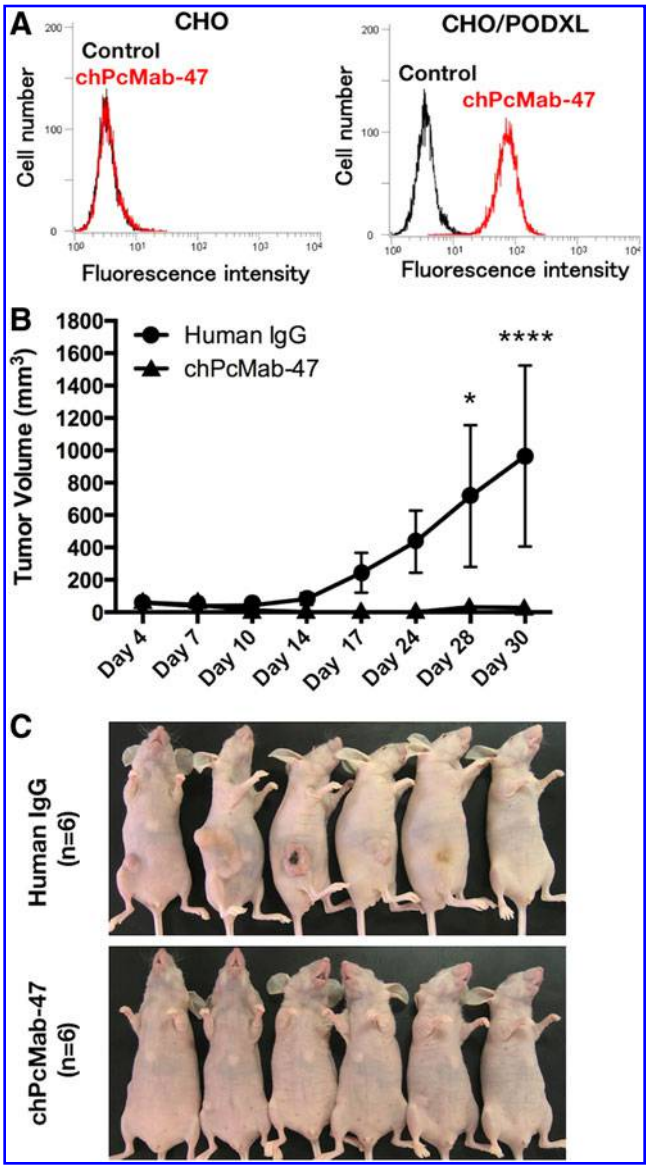

FIG. 3. Antitumor activity of chPcMab-47 against CHO/PODXL. **(A)** CHO-K1 and CHO/PODXL cells were treated with chPcMab-47 (1 μg/mL; red) or control PBS (black) for 30 minutes at 4°C, followed by treatment with FITC-labeled antihuman IgG. **(B)** CHO/PODXL cells (5×10^6 cells/100 μL) were subcutaneously inoculated into BALB/c nude mice. After 1 day, 100 μg of chPcMab-47 or control human IgG was injected into the peritoneal cavity of the mice. The antibodies were further injected four times (days 1, 4, 11, and 18; control group: $n = 6$; chPcMab-47 group: $n = 6$). NK cells were injected around the tumor on days 4 and 11. The tumor diameter was measured at 3- to 4-day intervals and was calculated using the following formula: tumor volume = $W^2 \times L/2$, where W is short diameter and L is long diameter. *$p < 0.05$, ****$p < 0.001$ with two-way ANOVA. **(C)** Comparison of the tumor size (day 30). ANOVA, analysis of variance; CHO, Chinese hamster ovary; NK, natural killer.

Results

Production of human–mouse chimeric antibody, chPcMab-47

We first checked the sensitivity and specificity of chPcMab-47 using flow cytometry. As depicted in Figure 1A, chPcMab-47 recognized endogenous PODXL, which is expressed in LN229 cells (a glioblastoma cell line), whereas it did not react with LN229/PODXL-KO cells (PDIS-13) (Fig. 1B), indicating that chPcMab-47 is specific against PODXL. The reaction of chPcMab-47 against LN229/PODXL was higher than that against LN229 (Fig. 1A). We next performed a kinetic analysis of the interaction of chPcMab-47 or PcMab-47 with LN229/PODXL using flow cytometry. As shown in Figure 2, K_D of chPcMab-47 was determined to be 1.1×10^{-8} M, indicating that chPcMab-47 possesses higher affinity against PODXL than PcMab-47 (K_D: 2.9×10^{-8} M).

Antitumor activity of chPcMab-47 against CHO/PODXL

chPcMab-47 reacted with CHO/PODXL but did not react with CHO-K1 (human PODXL-negative cells) (Fig. 3A). To investigate the antitumor activity of chPcMab-47 on primary tumor growth *in vivo*, CHO/PODXL cells were subcutaneously implanted into the flanks of nude mice. chPcMab-47 and control human IgG were injected four times (on days 1, 4, 11, and 18 after cell injections) into the peritoneal cavity of mice, and human NK cells were injected twice (on days 4 and 11) around the tumors. Tumor formation was observed in mice from the control and treated groups. However, chPcMab-47 significantly reduced tumor development compared with control human IgG on day 30 (Fig. 3B and C).

Antitumor activity of chPcMab-47 against colorectal cancers

PODXL is expressed in several cancers such as colorectal cancers[16–19] and breast cancers.[22–24] We performed flow cytometry using colorectal cancer cells, which revealed that chPcMab-47 reacted with the colorectal cancer cell lines HCT-15, Caco-2, HCT-5, and DLD-1 (Fig. 3). We selected HCT-15 because of its tumorigenicity *in vivo*.[25–27] To study the antitumor activity of chPcMab-47 on primary tumor growth *in vivo*, HCT-15 cells were subcutaneously implanted into the flanks of nude mice. chPcMab-47 and control human IgG were injected four times into the peritoneal cavity of mice, and human NK cells were injected twice around the tumors. Tumor formation was observed in mice from the control and treated groups. However, chPcMab-47 significantly reduced tumor development compared with control human IgG on day 29 (Fig. 4B and C).

Discussion

In our previous study, we immunized mice with recombinant PODXL, which was purified from the culture supernatant

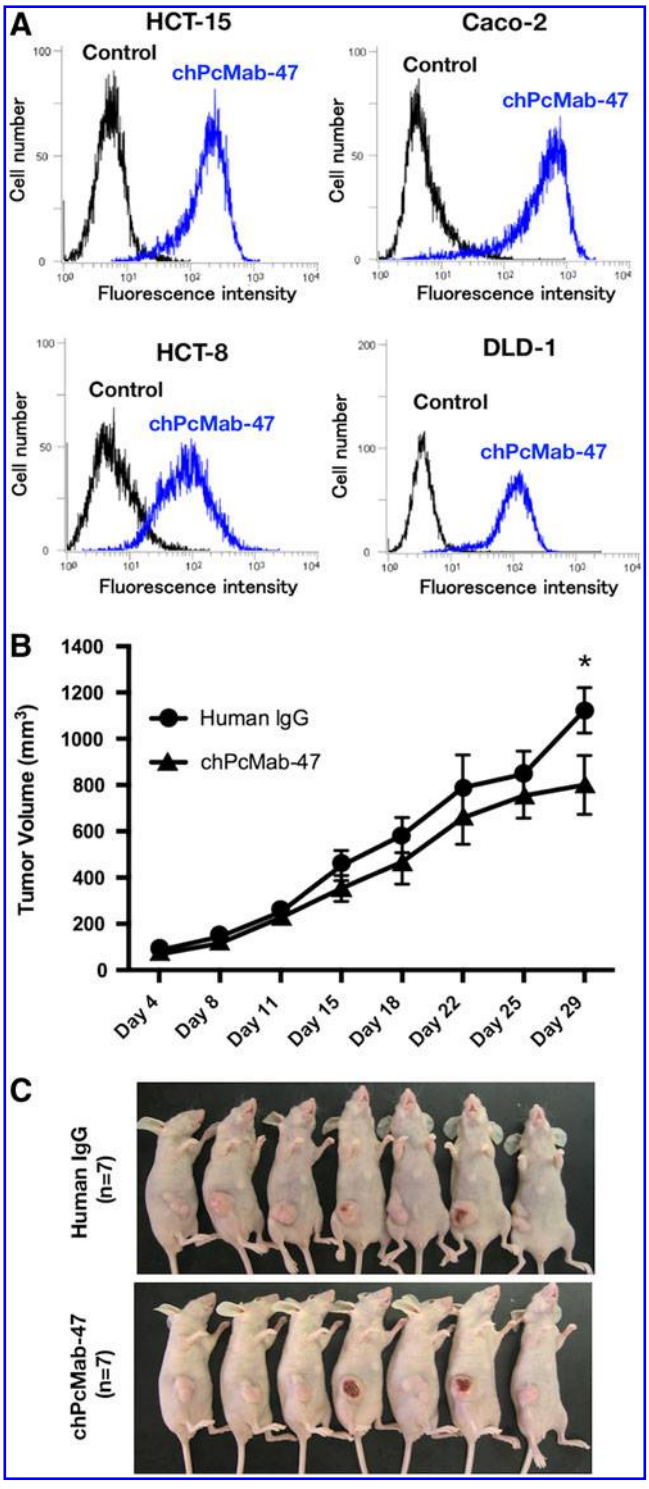

FIG. 4. Antitumor activity of chPcMab-47 against HCT-15 cells. **(A)** HCT-15, Caco-2, HCT-8, and DLD-1 cells were treated with chPcMab-47 (1 μg/mL; blue) or control PBS (black) for 30 minutes at 4°C, followed by treatment with FITC-labeled antihuman IgG. **(B)** HCT-15 cells (5×10^6 cells/100 μL) were subcutaneously inoculated into BALB/c nude mice. After 1 day, 100 μg of chPcMab-47 or control human IgG was injected into the peritoneal cavity of the mice. The antibodies were further injected four times (days 1, 7, 14, and 21; control group: $n = 7$; chPcMab-47 group: $n = 7$). NK cells were injected around the tumor on days 7 and 14. The tumor diameter was measured at 3- to 4-day intervals and was calculated using the following formula: tumor volume = $W^{2\times} L/2$, where W is short diameter and L is long diameter. $*p < 0.05$ with two-way ANOVA. **(C)** Comparison of the tumor size (day 29).

of LN229/ectodomain-PODXL.[21] One clone, PcMab-47 (mouse IgG_1, kappa) was successfully produced. PcMab-47 reacted with CHO/PODXL and LN229 cells (a glioblastoma cell line) but did not react with CHO-K1 cells (human PODXL-negative cells) and LN229/PODXL-KO cells (PDIS-13), indicating that PcMab-47 is specific against PODXL. PcMab-47 is also useful for immunohistochemical analysis against human colorectal adenocarcinomas and breast cancers.[21] In this study, we produced a human–mouse chimeric antibody from PcMab-47 and investigated whether chPcMab-47 possesses antitumor activity.

We investigated whether chPcMab-47 reacts with colorectal cancer cell lines. As shown in Figure 4, chPcMab-47 reacted with HCT-15, Caco-2, HCT-5, and DLD-1 cells at a low concentration of 1 μg/mL. We further checked the reactivity of chPcMab-47 against other colorectal cancer cell lines. It was observed that chPcMab-47 showed a moderate reaction with LST147T and SW1116 cells, a slight reaction with HT29 and HCT-116 cells, and no reaction with colo201 and colo205 cells, indicating that 80% (8/10) of cells were more or less detected by chPcMab-47 (data not shown). We searched the literature to find out whether those cell lines grow subcutaneously in BALB/c nude mice, and we discovered that HCT-15 is the best cell line for investigating antitumor activity.[25–27] In this study, we injected human NK cells around the tumors because chPcMab-47 needs to induce ADCC activity.[28,29] Although chPcMab-47 significantly reduced tumor development compared with control human IgG on day 29 (Fig. 4B), the antitumor activity of chPcMab-47 was not adequate for antibody-based target therapy. This result suggests that we need to combine chPcMab-47 with anticancer drugs or apply it to novel antitumor reagents, including T cells and viruses, to exert antitumor activity against cancer cells.

Taken together, chPcMab-47 could be useful for antibody therapy against PODXL-expressing cancers. PODXL is known to be expressed in the kidney, heart, pancreas, and breast tissues and plays an important role in these tissues,[9] indicating that we need to develop cancer-specific mAbs in the near future.

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Author Disclosure Statement

No competing financial interests exist.

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