MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 43, Number 2, 2024

DOI: 10.1089/mab.2024.0003

PMab-314: An Anti-Giant Panda Podoplanin Monoclonal Antibody

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The giant panda (*Ailuropoda melanoleuca*) is one of the important species in worldwide animal conservation. Because it is essential to understand the disease of giant panda for conservation, histopathological analyses of tissues are important to understand the pathogenesis. However, monoclonal antibodies (mAbs) against giant panda-derived proteins are limited. Podoplanin (PDPN) is an essential marker of lung type I alveolar epithelial cells, kidney podocytes, and lymphatic endothelial cells. PDPN is also overexpressed in various human tumors, which are associated with poor prognosis. Here, an anti-giant panda PDPN (gpPDPN) mAb, PMab-314 (mouse IgG_1, kappa) was established using the Cell-Based Immunization and Screening method. PMab-314 recognized N-terminal PA16-tagged gpPDPN-overexpressed Chinese hamster ovary-K1 cells (CHO/PA16-gpPDPN) in flow cytometry. The K_D value of PMab-314 for CHO/PA16-gpPDPN was determined as 1.3×10^{-8} M. Furthermore, PMab-314 is useful for detecting gpPDPN in western blot analysis. These findings indicate that PMab-314 is a useful tool for the analyses of gpPDPN-expressed cells.

Keywords: giant panda, podoplanin, monoclonal antibody, flow cytometry, western blotting

Introduction

The giant panda (*Ailuropoda melanoleuca*) is an endangered animal in the world. The Fourth National Survey Report on the Giant Panda reported that there are 1864 wild giant pandas in 33 segregated local populations. However, 25 of these populations are too small to be self-sustaining and are at high risk of extinction.[1] The main causes of death of giant pandas were gastrointestinal diseases, parasites, and viral infections.[2,3] With an increased population of captive-bred giant pandas, chronic kidney disease (CKD)[4] and tumor[5,6] in the giant pandas were also reported. These reports showed the histological analysis of affected kidney,[4] pancreatic ductal adenocarcinoma (PDAC),[5] and testicular seminoma.[6]

Podoplanin (PDPN) is a type I transmembrane mucin-like protein[7] conserved in various species[8] and is an important marker of lung type I alveolar cells,[9,10] kidney podocytes,[7] and lymphatic endothelial cells.[11,12] In humans, PDPN is overexpressed in tumors and plays crucial roles in tumor invasion and metastasis.[13] Therefore, anti-PDPN monoclonal antibodies (mAbs) are useful to distinguish the specific cell types and investigate the PDPN-expressing tumor cells.

We have developed mAbs against PDPN of various species including human,[14] mouse,[15] rat,[16] rabbit,[17] dog,[18] bovine,[19] cat,[20] tiger,[21] horse,[22] pig,[23] goat,[24] sheep,[25] alpaca,[26] Tasmanian devil,[27] bear,[28] whale,[29] California sea lion,[30] hamster,[31] ferret,[32] elephant,[33] and giraffe.[34] Here, we established an anti-giant panda PDPN (gpPDPN) mAb, PMab-314, using the Cell-Based Immunization and Screening (CBIS) method and evaluated the applications.

Materials and Methods

Preparation of cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). The full-length open reading

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frame of gpPDPN (Accession No.: XM_011232779) was synthesized by Eurofins Genomics KK. The gpPDPN sequence, in which the original signal sequence of the N-terminus ($_1$-MWKVPVLLLVLGSVWLWDLAEG-$_{22}$) was deleted, was obtained by polymerase chain reaction. Interleukin 2-signal sequence and PA16 tag or MAP16 tag of the N-terminus of gpPDPN were inserted into pCAGzeo vectors. The amino acid sequences of the PA16 tag and the MAP16 tag were GLEGGVAMPGAEDDVV and PGTGDGMVPPGI EDKI, respectively. The PA16 tag and the MAP16 tag can be detected by NZ-1[14,35] and PMab-1,[15,36] respectively.

The gpPDPN plasmids were transfected into CHO-K1 cells, using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). Clones stably expressing gpPDPN were established through cell sorting using a cell sorter (SH800; Sony Corp., Tokyo, Japan), after which cultivation in a medium, containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA) was conducted.

CHO-K1, gpPDPN-overexpressed CHO-K1 (CHO/MAP16-gpPDPN and CHO/PA16-gpPDPN), and P3U1 cells were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). All cells were grown in a humidified incubator at 37°C, in an atmosphere of 5% CO_2 and 95% air.

Production of hybridomas

Two five-week-old BALB/c female mice were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under specific pathogen-free conditions. All animal experiments were performed according to the relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. The Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001) approved animal experiments.

To establish mAbs against gpPDPN, mice (aged six weeks) were intraperitoneally immunized with CHO/MAP16-gpPDPN cells (1×10^8 cells/mouse) plus Alhydrogel adjuvant 2% (InvivoGen). The procedure included three additional injections every week (1×10^8 cells/mouse), which was followed by a final booster intraperitoneal injection (1×10^8 cells/mouse), two days before harvesting splenocytes. The splenocytes were fused with P3U1 cells, using PEG1500 (Roche Diagnostics, Indianapolis, IN). Hybridomas were selected and cultivated in the RPMI-1640 medium with 10% FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B, 5 μg/mL of Plasmocin, 5% Briclone (NICB, Dublin, Ireland), and hypoxanthine, aminopterin, and thymidine (Thermo Fisher Scientific, Inc.). The supernatants were subsequently screened by flow cytometry using CHO/PA16-gpPDPN and CHO-K1.

Purification of PMab-314

The cultured supernatants of PMab-314-producing hybridomas were filtrated with Steritop (0.22 μm; Merck KGaA, Darmstadt, Germany) and subsequently applied to 1 mL of Ab-Capcher ExTra (ProteNova, Inc., Kagawa, Japan). After washing with phosphate-buffered saline (PBS), bound antibodies were eluted with an IgG elution buffer (Thermo Fisher Scientific, Inc.), followed by immediate neutralization of eluates, using 1M Tris-HCl (pH 8.0). Finally, the eluates were concentrated, after which the buffer was replaced with PBS using Amicon Ultra (Merck KGaA).

Flow cytometric analyses

CHO-K1 and CHO/PA16-gpPDPN cells were harvested by exposure to 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). The cells were washed with 0.1% bovine serum albumin in PBS and treated with PMab-314 (0.01–10 μg/mL) and NZ-1 (1 μg/mL) for 30 min at 4°C. Then, the cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA) for PMab-314 or Alexa Fluor 488-conjugated anti-rat IgG (1:1000; Cell Signaling Technology, Inc.) for NZ-1. The fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp.).

To determine the dissociation constant (K_D), PMab-314 was serially 1:1 diluted from 20 μg/mL to 1.2 ng/mL. Then, the cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:200). The geometric mean of fluorescence intensity of CHO/PA16-gpPDPN at each concentration was calculated by FlowJo (BD Biosciences, Franklin Lakes, NJ). The K_D was calculated by fitting saturation binding curves to the built-in; one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Western blotting

CHO-K1 and CHO/PA16-gpPDPN cells were harvested by exposure to 0.25% trypsin and 1 mM EDTA (Nacalai Tesque, Inc.). Cell lysates were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.), after which proteins were separated on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and transferred to polyvinylidene difluoride membranes (Merck KGaA). After blocking with 4% skim milk (Nacalai Tesque, Inc.) in 0.05% Tween 20-containing PBS, membranes were incubated with 1 μg/mL of PMab-314, an anti-PA16 tag mAb (NZ-1), or an anti-isocitrate dehydrogenase 1 (IDH1) mAb (RcMab-1).[37] Next, they were incubated again with horseradish peroxidase-conjugated anti-mouse immunoglobulins (1:1000; Agilent Technologies, Inc., Santa Clara, CA) for PMab-314 or horseradish peroxidase-conjugated anti-rat immunoglobulins (diluted 1:10,000; Sigma-Aldrich Corp., St. Louis, MO) for NZ-1 and RcMab-1. Finally, protein bands were detected using ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Results

Establishment of a novel anti-gpPDPN mAb

For the establishment of anti-gpPDPN mAb, we used the CBIS method. The CBIS method is composed of 2 steps: immunization of antigen-overexpressed cells and screening of hybridoma supernatants using flow cytometry. The CBIS method is a useful approach for the establishment of mAbs against glycoproteins[38,39] and multiple transmembrane proteins.[40–42] In this study, we immunized mice with CHO/MAP16-gpPDPN cells (Fig. 1A). The splenocytes from these

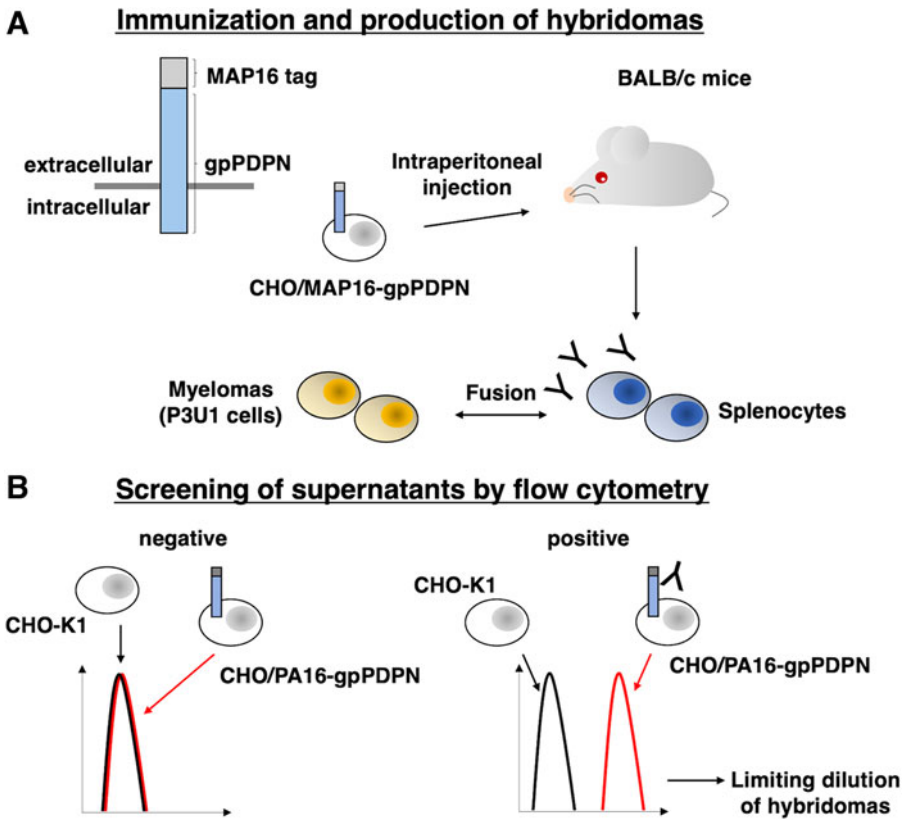

FIG. 1. The scheme of establishment of PMab-314 by the CBIS method. **(A)** Immunization and production of hybridomas. CHO/MAP16-gpPDPN cells were injected intraperitoneally into two BALB/c mice. The splenocytes were fused with P3U1 cells using polyethylene glycol. **(B)** Screening of hybridomas. The culture supernatants were screened through flow cytometry to select anti-gpPDPN mAb-producing hybridomas. PMab-314 was established by limiting dilution. CHO, Chinese hamster ovary; gpPDPN, giant panda podoplanin.

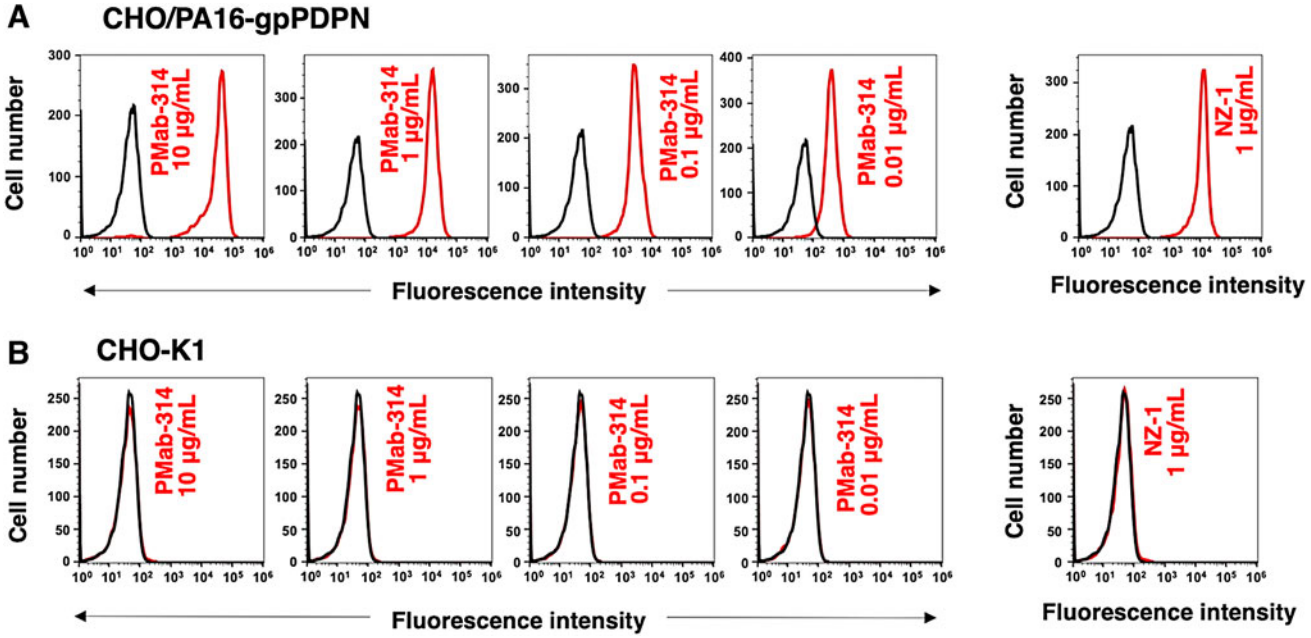

FIG. 2. Flow cytometry of gpPDPN-overexpressed cells using PMab-314. CHO/PA16-gpPDPN **(A)** and CHO-K1 **(B)** cells were treated with 0.01–10 µg/mL of PMab-314 (red line) or blocking buffer (negative control, black line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. NZ-1 is used as a positive control to detect PA16-gpPDPN expression on CHO/PA16-gpPDPN.

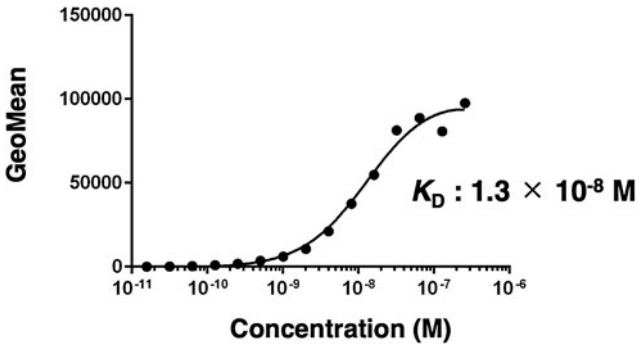

FIG. 3. The determination of the binding affinity of PMab-314 against CHO/PA16-gpPDPN cells by flow cytometry. The dots are the geometric mean of fluorescence intensity of CHO/PA16-gpPDPN at each concentration. The solid lines show the fitting curve calculated by GraphPad PRISM 6.

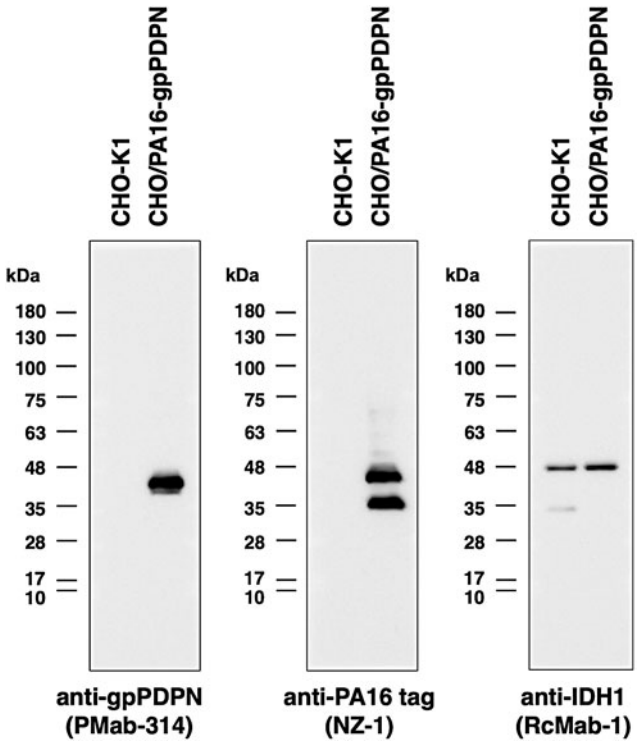

FIG. 4. Western blot analysis using PMab-314. Cell lysates (10 μg) of CHO-K1 and CHO/PA16-gpPDPN cells were electrophoresed, after which proteins were transferred to polyvinylidene difluoride membranes. After blocking, membranes were subsequently incubated with PMab-314, an anti-PA16 tag mAb (NZ-1), or an anti-IDH1 mAb (RcMab-1).

mice were fused with P3U1 cells by using polyethylene glycol. Hybridomas producing antibodies against CHO/PA16-gpPDPN cells were selected by flow cytometry (Fig. 1B). After limiting dilution of hybridomas, PMab-314 (mouse IgG_1, kappa) was established.

Flow cytometry using PMab-314

We examined the reactivity of PMab-314 to gpPDPN by flow cytometry. PMab-314 reacted to CHO/PA16-gpPDPN cells in a dose-dependent manner (Fig. 2A). In contrast, PMab-314 did not bind to CHO-K1 cells even at 10 μg/mL (Fig. 2B). These data indicated that PMab-314 specifically detects gpPDPN.

Next, we determined the K_D of PMab-314 with CHO/PA16-gpPDPN cells by flow cytometry. After the calculation of the geometric mean at each concentration and fitting one-site binding models, the K_D value of PMab-314 for gpPDPN was determined as 1.3×10^{-8} M (Fig. 3).

Western blotting using PMab-314

Finally, we examined the application of PMab-314 in western blotting. Lysates of CHO-K1 and CHO/PA16-gpPDPN cells were probed. PMab-314 and NZ-1 detected the 48-kDa band of gpPDPN in lysates from CHO/PA16-gpPDPN cells, whereas any bands were not detected in lysates of CHO-K1 cells (Fig. 4).

Discussion

CKD exhibits irreversible and progressive loss of function of the kidney. Previous reports showed that CKD was a common cause of mortality in captive animals such as bears.[43] In giant panda, a case report describes clinical features of azotemia, polyuria, and anemia.[4] The histopathological analysis of kidney showed that glomeruli showed diffuse cystic changes with capillary tufts atrophy, and Bowman's capsules were moderately thickened.[4] Furthermore, there was marked interstitial fibrosis which replaced approximately half of glomeruli and tubules.[4] Since PDPN is expressed in podocytes in the glomeruli, PMab-314 could contribute to the analysis of podocytes in the kidney.

Similarities between tumors developed in giant pandas and humans have been reported. PDAC was discovered in a captive adult female giant panda.[5] Immunohistochemical staining showed that ductal adenocarcinoma cells in the giant panda expressed cytokeratins 7 and 19, which are expressed as 90% of PDAC in humans.[44,45] Cancer-associated fibroblasts (CAFs) are one of the most significant components in the tumor microenvironment of PDAC.[46] PDPN expression in CAFs enhances tumor progression of PDAC in humans.[47] In 2023, a captive adult male giant panda was diagnosed with testicular seminoma and underwent surgical management.[6] The authors diagnosed testicular seminoma due to the elevation of testicular tumor markers including α-fetoprotein and lactate dehydrogenase, which are markers of testicular tumor in humans.[48] We previously reported that PDPN is an important diagnostic marker that distinguishes seminoma from embryonal carcinoma in human testicular germ cell tumors.[49] PMab-314 could also contribute to the diagnosis of testicular seminoma in giant panda.

Authors' Contributions

T.O., G.L., M.Y., T.N., S.H., and T.T. performed the experiments. M.K.K. and Y.K. designed the experiments. T.O. and T.T. analyzed the data. T.O., H.S., and Y.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported, in part, by Japan Agency for Medical Research and Development (AMED) under Grant Nos.: JP23ama121008 (to Y.K.), JP23am0401013 (to Y.K.), JP23bm1123027 (to Y.K.), and JP23ck0106730 (to Y.K.), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) Grant Nos.: 23K19494 (to T.O.), 21K20789 (to T.T.), 22K06995 (to H.S.), 21K07168 (to M.K.K.), and 22K07224 (to Y.K.).

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Received: February 1, 2024
Accepted: February 24, 2024