**Supplemental Materials and Methods**

**Development and maintenance of patient-derived OS cells**

Clinical specimens were obtained from patients with OS (see the patient information in IHC staining section) at JFCR Hospital with informed consent as per the protocol approved by the IRB committee of the JFCR. The OS clinical samples were subcutaneously engrafted into NSG or NOD-SCID mice to obtain patient-derived xenograft models. Sa-xeno-147-P0 cell line was developed from a patient-derived xenograft tumor and was cultured in a collagen-coated dish with RPMI1640/Ham’s F12 (1:1) medium containing 15% FBS, 20 mM HEPES, and 1× Antibiotic Antimycotic Solution (Penicillin/Streptomycin/Amphotericin B) (Sigma-Aldrich). GCB53-G3 was developed from a xenograft tumor derived from GCB53 cells, which was developed directly from a clinical sample. GCB53-G3 was cultured in RPMI1640 medium containing 10% FBS and 250 μg/ml kanamycin.

**Cell culture conditions**

OS cell lines were cultured in specific media: SJSA-1, NOS-10, and HOS in RPMI1640 (Wako, Osaka, Japan); 143B, MNNG/HOS Cl #5, KHOS/NP, and NY in Minimum Essential Medium (Sigma-Aldrich, St Louis, MO, USA or Nacali Tesque, Kyoto, Japan); MG63 and HOS in Dulbecco’s modified Eagle’s medium (Wako); and Saos-2 in McCoy’s 5A medium (Sigma-Aldrich). All media were supplemented with 10% FBS and 250 μg/ml kanamycin (Meiji Seika Pharma, Tokyo, Japan).

**Immunoblot analysis**

Cell lysates were prepared by suspension in lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM EDTA) and boiled. The supernatants were collected from the cell lysates by centrifugation at 10,000 × *g* for 10 min and was used to examine the protein concentration with BCA protein assay reagent (Thermo) with electrophoresis in an SDS-PAGE gel. Protein was then submarine-transferred to PVDF membranes and immunoblotted with antibodies (Abs) against human PDPN (PG4D2), PDGFRα (Cell Signaling Technology; CST), PDGFRβ (CST), phospho-PDGFRα/β (Thermo), phospho-Akt (S473, CST), Akt, or β-actin (clone: AC-15, Sigma). HRP-conjugated anti-mouse or rabbit IgG and enhanced chemiluminescence reagent (ECL prime, GE Healthcare) were used for detection. The chemiluminescence signals were detected by the image analyzer AI600 (GE Healthcare).

**Human phospho-RTK array**

Phosphorylation of RTKs in SJSA-1 cells treated with non-activated or activated platelet supernatants was estimated using the human phospho-RTK array kit (R & D Systems). Briefly, SJSA-1 cells were co-cultured with control or SJSA-1-activated platelet supernatants for 15 min. The cell lysates (300 µg) were then incubated with each human phospho-RTK array according to the manufacturer’s protocols. The chemiluminescence signal was detected using image analyzer AI600 (GE Healthcare). The signal intensities of duplicated spots were quantified and normalized against the signal of positive control spots.

**Flow cytometry**

To detect PDPN expression, the harvested cells were treated with 1 μg/ml of anti-PDPN Ab D2-40 or control mouse IgG2a (Sigma-Aldrich), followed by incubation with 4 μg/mL of Alexa Fluor 488-conjugated anti-mouse IgG (H + L) (Thermo Fisher Scientific). To detect the interaction between cell surface expressed PDPN and CLEC-2, the cells were incubated with 0.4 μg/mL of Hisx10-tagged human CLEC-2 protein (R & D Systems), followed by incubation with Alexa Fluor 488-conjugated anti-penta-His Ab (1:500 dilution) (QIAGEN). Flow cytometric analyses detecting the Alexa signal on the cells were performed using the Cytomics FC500 flow cytometer system (Beckman Coulter).

**Establishment of chimeric humanized PG4D2 (chPG4D2) Ab**

The generation of chimeric Abs has been described previously (1,2). In brief, the cDNA of the H and L chains of PG4D2 mAb were sequenced and analyzed by the online V-Quest software provided by the International ImMunoGeneTics database (http://www.imgt.org/IMGT\_vquest/share/textes/) to identify CDRs. The CDR fragments of the H and L chains were subcloned into the H or kappa L chain constant regions of human IgG4 and constructed the expression vector. Using the virus expression vector, expressed and secreted chimeric Abs into media from CHO were purified to be used for in vitro assay.

**Construction of humanized Ab-expressing vectors**

Humanized AP201 Ab was generated by grafting CDRs of murine PG4D2 monoclonal antibody (mAb) into human frameworks(3,4). Briefly, murine PG4D2 mAb CDR canonical structures were determined, and the most homologous human V and J germline genes for either H or L chain variable regions (VH or VL, respectively) of murine Abs were identified using IMGT analysis tools (http://www.imgt.org). For the H chain, the human germline genes *IGHV3-7\*01* or *IGHV3-66\*01* for V genes and *IGHJ1\*01* for J gene, exhibited the highest homology to their murine counterparts, sharing 86% and 91% sequence identities, respectively. For the L chain, the human *IGKV2-28\*01* and *IGKJ4\*01* genes displayed high homologies of 84% and 80%, respectively, with their murine counterparts. Framework sequences of these selected human germline genes were used as acceptor sequences for murine PG4D2 mAb CDRs. Furthermore, the humanized VH and VL genes were grafted onto human γ4 H chain constant region and human κ LC constant region, respectively, to assemble the complete humanized Ab genes. The nucleotide sequence of human γ4 H chain constant region was modified so that the corresponding amino acid sequence of the Cys-Pro-Ser-Cys core hinge of IgG4 was changed to Cys-Pro-Pro-Cys (IgG4SP). The nucleotide sequences of the entire H chain and L chain were codon-optimized for expression in CHO cells and synthesized by GenScript Japan (Tokyo, Japan) and Eurofins genomics K.K. (Tokyo, Japan), respectively.

**Expression and purification of AP201 Ab**

To express the humanized Ab, the following two procedures were performed.

1. Synthetic genes of H chain and L chain were inserted into a Freedom pCHO 1.0 plasmid (Thermo Fisher Scientific), and the plasmid was linearized and transfected into Freedom CHO-S cells using the lipophilic agent, FreeStyle MAX (Thermo Fisher Scientific). Forty-eight hours after transfection, the cells were propagated in CD FortiCHO medium containing 40 μg/mL puromycin and 0.2 μmol/L MTX (Sigma-Aldrich) in the first phase, and 100 μg/mL puromycin and 1 μmol/L MTX in the second phase. The selected pools were serially diluted and seeded into 96-well plates, and then positive pools were re-selected by evaluating cell density and productivity using ELISA.
2. Synthetic genes of H chain and L chain were inserted into a modified pOptiVEC plasmid (Thermo Fisher Scientific), and the plasmid was linearized and transfected into CHO-DG44 cells (Thermo Fisher Scientific) by Nucleofection using the SG Cell Line 4D –Nucleofector X Kit L (LONZA). Forty eight hours after transfection, the cells were propagated in CD OptiCHO medium without hypoxanthine and thymidine. MTX (Sigma-Aldrich) concentration was increased stepwise to a final concentration of 4 μmol/L. High-producing clones were selected by Fluorescence-activated cell sorting using SH800 Cell Sorter (Sony Co.). The high producer clones were determined by ELISA.

After the selection steps, the cells produced by method (i) were cultured in a fed-batch mode in a stirred bioreactor with BalanCD CHO Growth A medium (FUJIFILM Irvine Scientific) containing 8 mmol/L glutamine at a stirring rate of 100 rpm, pH 7.0, at a temperature of 37.0°C and 60% dissolved oxygen. Feeding with the same medium started on day 3 at a fixed rate of 3% of the initial working volume, which was repeated every two days. The temperature was changed to 30.0°C when the cells reached a density of 8 × 106 cells/mL, and the pH was maintained at 7.0 with 10% (w/v) sodium carbonate. Cultivation was terminated on day 8 when cell viability reached ≤70%. The cells produced by method (ii) were cultured in a fed-batch mode in a stirred bioreactor with BalanCD CHO Growth A medium (FUJIFILM Irvine Scientific) containing 8 mmol/L glutamine at a stirring rate of 100 rpm, pH 7.0, at a temperature of 37.0°C and 60% dissolved oxygen. Feeding with BalanCD CHO Feed 4 (FUJIFILM Irvine Scientific) started on day 3 at a fixed rate of 4% of the initial working volume, which was repeated every two days. Glucose was added to a final concentration of 8 g/L when the glucose concentration reached below 3 g/L. Cultivation was terminated on day 11.

The harvested culture by both methods was clarified by Millistak+ Pod depth filters D0HC and A1HC (Merck Millipore). Antibodies were purified using MabSelect SuRe protein A agarose (GE Healthcare). The eluted antibodies were then concentrated and diafiltrated against 20 mmol/L sodium phosphate buffer with 150 mmol/L sodium chloride at pH 7.0. After adding TWEEN 80 to a final concentration of 0.1% (w/w), the Abs were filtrated by a 0.22-μm Millex-GV sterile filter.

**Estimation of binding activity of Abs to the PLAG4 domain of human PDPN by ELISA**

The PLAG4 domain peptide, N-CTGIRIEDLPTSEST-C, was immobilized on 96-well plates. After blocking with 4% (w/v) Block Ace solution (KAC, UKB80), the plates were incubated with the humanized mAb to CLEC-2 or control human IgG4. The plates were then incubated with a biotinylated goat anti-human IgG-Fc Ab (Abcam). Finally, the plates were incubated with streptavidin-β-galactosidase conjugate (Roche) followed by addition of its substrate, 4-methylumbelliferyl-β-D-galactopyranoside (FUJIFILM Wako). Fluorescence was measured at an excitation wavelength of 355 nm with an emission wavelength of 460 nm.

**Detection of inhibitory activity of Abs against CLEC-2−PDPN binding by ELISA**

Recombinant human CLEC-2 was immobilized on 96-well plates. After blocking, the plates were incubated with recombinant human PDPN-Fc chimera (R & D Systems) in the presence of PG4D2 mAb, the humanized mAb to CLEC-2 or control human IgG4. The plates were incubated with a biotinylated goat anti-human IgG-Fc Ab. Finally, the plates were incubated with streptavidin-β-galactosidase conjugate followed by addition of its substrate, 4-methylumbelliferyl-β-D-galactopyranoside. Fluorescence was measured at an excitation wavelength of 355 nm with an emission wavelength of 460 nm.

**Estimation of ADCC and CDC assays**

ADCC was analyzed using Jurkat cells transfected with expression vectors of FcγRIIIa and NFAT response elements with the luciferase gene as the effector cell (Promega) and SJSA-1 as the PDPN, EGFR-positive target cell. The SJSA-1 cells (1.25 × 104 cells/well) were seeded into a 96-well plate. The effector cells (7.5 × 104 cells/well) and titrated Abs were added into the wells. After 6 h of culture, detection reagent was added and the luminescence signals were monitored by a plate reader (Spectra Max iD3, Molecular Device). CDC was analyzed using baby rabbit complement (Cedarlane) and SJSA-1. The SJSA-1 cells (5 × 103 cells/well) were seeded into a 96-well plate. On the next day, Ab (1 μg/ml), and complement (1:20) were added into the wells, and after 5.5 h of culture, LDH-detection reagent (Promega) was used to detect cell lysis. The signal was detected by measuring OD492 nm−OD690 nm with a plate reader (Multiskan GO, Thermo).

**Surface Plasmon Resonanceanalysis**

Surface Plasmon Resonance (SPR) analysis of antibody was performed using a Biacore X100 (GE healthcare) as described in (5). Recombinant human podoplanin-Fc protein (R & D Systems, Minneapolis, MN, USA) was immobilized on a CM5 sensor chip (GE Healthcare). Final levels of immobilization were approximately 540.1 or 600.6 response units (measure conditions of AP201, or PG4D2, respectively). Five concentrations of human IgG4, AP201, or PG4D2 were flowed over the chip in the single cycle kinetics (3.7, 11.1, 33.3, 100, 300 nM, flow: 30 μl/min, contact time: 60 sec, dissociation time: 1800 sec). Sensorgrams were fit by global analysis using the Biacore X100 evaluation software. Efforts toward determining the equilibrium dissociation constant (KD) were decided using the bivalent binding analyte model.

**Safety study using *PdpnKI/KI* mice**

*PdpnKI/KI* C57BL/6N mice (n = 6 per group), which were 9 weeks of age and weighed 22.6–27.4 g, were intravenously injected with 50 mg/kg of AP201, control human IgG4, or vehicle PBS (5 ml/kg) through the tail vein. Body weight was measured 3 and 7 d after injection. Blood was collected 3 and 7 d after the injection for calculation of half-time in blood. Concentration of antibody in the serum was analyzed by ELISA. Blood collected 7 d after the injection was used for hematology and biochemical analysis. The mice were euthanized 2 weeks after injection, and tissues and organs were weighed. Most of the organs and tissues were fixed in 10% neutral buffer formalin, and the testis, epididymis, and eyes were fixed in modified Dabidson solution. All specimens were stored in 10% neutral buffer formalin, were paraffin-embedded, sliced, and stained with hematoxylin-eosin in accordance with the standard method. The treatment and analysis of specimens by a pathologist were performed at Nihon Bioresearch.

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