LpMab-19 Recognizes Sialylated *O*-Glycan on Thr76 of Human Podoplanin

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Human podoplanin (hPDPN) is expressed in lymphatic vessels, pulmonary type-I alveolar cells, and renal glomerulus. The hPDPN/C-type lectin-like receptor-2 (CLEC-2) interaction is involved in platelet aggregation and cancer metastasis. High expression of hPDPN in cancer cells or cancer-associated fibroblasts (CAFs) leads to a poor prognosis for cancer patients. In our previous research, we reported on several anti-hPDPN monoclonal antibodies (mAbs), including LpMab-2, LpMab-3, LpMab-7, LpMab-9, LpMab-12, LpMab-13, and LpMab-17 of mouse IgG_1 subclass, which were produced using CasMab technology. Here we produced a novel anti-hPDPN mAb LpMab-19 of mouse IgG_{2b} subclass. Flow cytometry revealed that the epitope of LpMab-19 includes O-glycan, which is attached to Thr76 of hPDPN. We further identified the minimum epitope of LpMab-19 as Thr76–Arg79 of hPDPN. Immunohistochemistry revealed that LpMab-19 is useful for detecting not only normal cells, including lymphatic vessels, but also glioblastoma and oral squamous cell carcinoma cells. LpMab-19 could be useful for investigating the physiological function of O-glycosylated hPDPN.

Keywords: podoplanin, PDPN, CasMab, glycopeptide, monoclonal antibody

Introduction

To determine the physiological function of membrane proteins in normal tissues and cancers, it is necessary to have highly sensitive and specific monoclonal antibodies (mAbs). Human podoplanin (hPDPN), known as a platelet aggregation-inducing (PLAG) factor (gp44/Aggrus), is involved in cancer metastasis.[1–10] We have produced many anti-hPDPN mAbs,[11–21] including NZ-1, which was produced by immunizing rats with the synthetic peptide (hpp3851) of hPDPN, and it has high specificity, sensitivity, and binding affinity for hPDPN.[21–23] The high binding affinity of NZ-1 has also been confirmed by other studies.[24,25] NZ-1 was highly internalized into glioma cell lines and also well accumulated into tumors *in vivo*; therefore, NZ-1 is a suitable candidate for therapy against malignant gliomas.[23,26] NZ-1 has antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity against hPDPN-expressing tumor cells.[27] Furthermore, NZ-1 inhibited the tumor cell-induced platelet aggregation and tumor metastasis by its neutralizing activity.[28] NZ-1 possesses good applications in many experiments, such as western blot, flow cytometry, immunohistochemical analysis, and immunoprecipitation.

We recently established the CasMab technology, which produces cancer-specific mAbs, although one protein in the cancer and normal cells has the same amino acid sequence.[11] We used LN229/hPDPN cells, not purified recombinant proteins, for immunization to develop novel anti-hPDPN mAbs.

Although we have produced many anti-hPDPN mAbs using CasMab technology,[11–17,19,20] almost all mAbs, including LpMab-2, LpMab-3, LpMab-7, LpMab-9, LpMab-12, LpMab-13, and LpMab-17, were determined to belong to the IgG_1 subclass. Only the clone LpMab-10 was classified as IgG_3 subclass; however, mouse IgG_3 subclass is often aggregated.[29] Furthermore, almost all anti-hPDPN mAbs react with PLAG domains (PLAG1–PLAG3) of hPDPN.[21,22,30–33] A novel PLAG domain of hPDPN was recently reported as PLAG4 ($_{81}$-EDLPT-$_{85}$), and anti-PLAG4 mAbs were produced.[34] We need to produce further anti-hPDPN mAbs, which target different epitopes of hPDPN, to fully understand the pathophysiological function of hPDPN.

Here we produced a novel anti-hPDPN mAb, LpMab-19 (mouse IgG_{2b}, kappa), the epitope of which is a O-glycosylated glycopeptide of hPDPN.

Materials and Methods

Cell lines

LN229, HEK-293T, Chinese hamster ovary (CHO)-K1, and P3U1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). LN319 was donated by Prof. Kazuhiko Mishima (Saitama Medical University, Saitama, Japan).[35] CHO-S was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). LN229 was previously transfected with hPDPN plasmids (LN229/hPDPN) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according

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to the manufacturer's instructions.[11] The HEK-293T/ hPDPN-knockout (KO) cell line (PDIS-2) and the LN319/ hPDPN-KO cell line (PDIS-6) were generated by transfection using CRISPR/Cas plasmids (Target ID: HS0000333287) that target PDPN (Sigma-Aldrich, St. Louis, MO). PDIS-2 and PDIS-6 cells were screened using the NZ-1 mAb.[12,15,18–20]

CHO-K1 and P3U1 were cultured in RPMI 1640 medium, including L-glutamine (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). CHO-S was cultured using CHO-S-SFM II (Thermo Fisher Scientific, Inc.). LN229, LN229/hPDPN, LN319, HEK-293T, PDIS-2, and PDIS-6 were cultured in Dulbecco's modified Eagle's medium, including L-glutamine (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Antibiotics, including 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 25 µg/mL of amphotericin B (Nacalai Tesque, Inc.), were added to all media.

Production of deletion mutants

The amplified *hPDPN* complementary DNA (cDNA) was subcloned into a pCAG-Ble(Zeo) vector (Wako Pure Chemical Industries Ltd. [Osaka, Japan]) with an MAP tag, which was detected by PMab-1[36,37] and added at the N-terminus using In-Fusion HD cloning kit (Clontech Laboratories Inc., Mountain View, CA). Deletion mutants of *hPDPN* were produced using several primers. Sense primers used were as follows: AGAAGACAAAAAGCTTGCCAGCACAGGCC AGCC for dN23, AGAAGACAAAAAGCTTGAAGGCG GCGTTGCCAT for dN37, AGAAGACAAAAAGCTTGCC GAAGATGATGTGGTG for dN46, AGAAGACAAAAA GCTTACCAGCGAAGACCGCTA for dN55, AGAAGAC AAAAAGCTTACAACTCTGGTGGCAACA for dN64, AG AAGACAAAAAGCTTGTAACAGGCATTCGCATC for dN75, AGAAGACAAAAAGCTTACTTCAGAAAGCACA GTCC for dN85, AGAAGACAAAAAGCTTCAAAGTCC AAGCGCCAC for dN95, AGAAGACAAAAAGCTTGCC ACCAGTCACTCCAC for dN105.

The following antisense primer was used: TCTAGAG TCGCGGCCGCTTACTTGTCGTCATCGT.

CHO-K1 cells were transfected with the plasmids using a Lipofectamin LTX (Thermo Fisher Scientific, Inc.). Deletion mutants were cultured in RPMI 1640 medium including L-glutamine supplemented with heat-inactivated FBS. Stable transfectants of CHO-K1/ssMAP-hPDPNdN mutants were selected by cultivating the transfectants in a medium containing 0.5 mg/mL of zeocin (InvivoGen, Inc., San Diego, CA).

Production of point mutants

The amplified *hPDPN* cDNA was subcloned into a pcDNA3 vector (Thermo Fisher Scientific, Inc.), and a FLAG epitope tag was added at the C-terminus. Amino acids in hPDPN were substituted with alanine or glycine using a QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA) with oligonucleotides containing the desired mutations. CHO-S or CHO-K1 cells were transfected with the plasmids using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc., Berke-

ley, CA). Point mutants of CHO-K1 and CHO-S were cultured in RPMI 1640 medium including L-glutamine supplemented with heat-inactivated FBS and CHO-S-SFM II, respectively.

Hybridoma production

Four-week-old female BALB/c mice (CLEA Japan, Tokyo, Japan) were immunized by intraperitoneal (i.p.) injection of 1×10^8 LN229/hPDPN cells together with Imject Alum (Thermo Fisher Scientific, Inc.).[11] After several additional immunizations, a booster i.p. injection was given 2 days before mice were euthanized by cervical dislocation, and spleen cells were harvested. The spleen cells were fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). Hybridomas were grown in RPMI 1640 medium including L-glutamine with hypoxanthine, aminopterin, and thymidine selection medium supplement (Thermo Fisher Scientific, Inc.). Culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA) for binding to recombinant hPDPN purified from LN229/hPDPN cells. Proteins were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at 1 µg/mL for 30 minutes. After blocking with 1% bovine serum albumin (BSA) in 0.05% Tween20/phosphate-buffered saline (PBS; Nacalai Tesque, Inc.), the plates were incubated with culture supernatant followed by 1:2000 diluted peroxidase-conjugated antimouse IgG (Agilent Technologies, Inc.). The enzymatic reaction was produced with a 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc.).

Flow cytometry

Cell lines were harvested by brief exposure to 0.25% trypsin/1 mM EDTA (Nacalai Tesque, Inc.). After washing with 0.1% BSA in PBS, cells were treated with primary mAbs for 30 minutes at 4°C, followed by treatment with Oregon Green 488 goat antimouse IgG or antirat IgG (Thermo Fisher Scientific, Inc.). Fluorescence data were collected using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

Immunohistochemical analyses

The use of human cancer tissues was reviewed and approved by Sendai Medical Center Review Board.[19] Written informed consent was obtained for the human cancer tissue samples used in this study. Four-micrometer-thick histologic sections were deparaffinized in xylene and rehydrated. After antigen retrieval procedure (autoclave using citrate buffer, pH 6.0; Agilent Technologies, Inc.), sections were incubated with 1 µg/mL of LpMab-19 for 1 hour at room temperature followed by treatment with Envision+ kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3,3-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.) for 5 minutes, and then the sections were counterstained with hematoxylin (Wako Pure Chemical Industries Ltd.).

Results and Discussion

The expression of hPDPN has been reported in many cancers, such as oral cancer, lung cancer, esophageal cancer, malignant mesothelioma, malignant glioma, testicular tumor,

bladder cancer, and osteosarcoma.[8,21,23,26,38–46] hPDPN is a PLAG factor, which is involved in cancer metastasis.[8–10] hPDPN-expressing cancer-associated fibroblasts are associated with poor prognosis of several cancers.[47–52] We previously identified C-type lectin-like receptor-2 (CLEC-2) as an endogenous receptor of hPDPN,[28,53] and recently performed comparative crystallographic studies on hPDPN in association with CLEC-2.[54] The interaction with CLEC-2 was mainly observed at Glu47 and Asp48 in the PLAG3 domain and the α2–6-linked sialic acid at Thr52 of hPDPN. Clone LpMab-12, our recently produced mAb, specifically detects this sialylated Thr52.[18]

Herein we used the CasMab technology for production of anti-hPDPN mAbs.[11] LN229/hPDPN cells were immunized into mice, and culture supernatants were screened using ELISA for binding to recombinant hPDPN, which was purified from LN229/hPDPN cells.[11] Finally, LpMab-19 (mouse IgG$_{2b}$, kappa), the first IgG$_{2b}$ of mouse anti-hPDPN mAbs using CasMab technology, was produced after limiting dilution.

LpMab-19 reacted with LN229/hPDPN, and did not with LN229 (hPDPN-negative cell) in flow cytometry (Fig. 1A). LpMab-19 recognized endogenous hPDPN, which is expressed in LN319 (a glioblastoma cell line), whereas it did
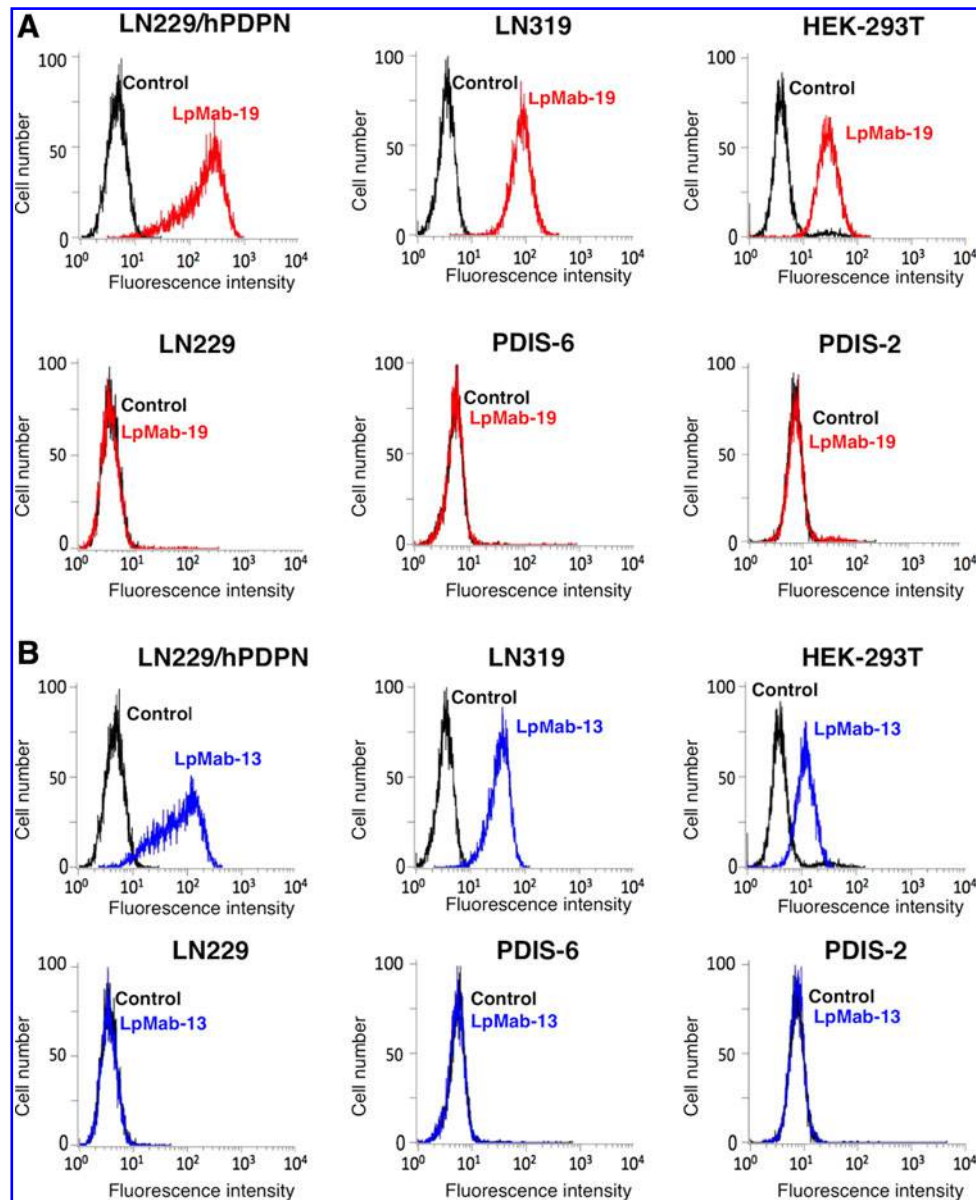

FIG. 1. In flow cytometry, LpMab-19 specifically detects hPDPN. (A) LN229, LN229/hPDPN, LN319, LN319/hPDPN-KO cell (PDIS-6), HEK-293T, and HEK-293T/hPDPN-KO cell (PDIS-2) were treated with LpMab-19 (1 μg/mL; red) or control PBS (black) for 30 minutes at 4°C, followed by treatment with antimouse IgG-Oregon green. (B) LN229, LN229/hPDPN, LN319, LN319/hPDPN-KO cell (PDIS-6), HEK-293T, and HEK-293T/hPDPN-KO cell (PDIS-2) were treated with LpMab-13 (1 μg/mL; blue) or control PBS (black) for 30 minutes at 4°C, followed by treatment with antimouse IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. hPDPN, human podoplanin; PBS, phosphate-buffered saline.

not react with LN319/hPDPN-KO cell (PDIS-6) (Fig. 1A). In addition, LpMab-19 detected hPDPN of HEK-293T (an epithelial cell line of kidney), but lost the reaction with HEK-293T/hPDPN-KO cell (PDIS-2) (Fig. 1A). As a positive control, an anti-hPDPN mAb LpMab-13 was used (Fig. 1B).

LpMab-13 detected LN229/hPDPN, LN319, and HEK-293T, whereas it lost the reaction with PDIS-6 and PDIS-2.

We herein produced several deletion mutants of hPDPN, which were expressed in CHO-K1 cell lines. LpMab-19 detected the delta N-terminus (dN)23 that starts from Ala23.

FIG. 2. Epitope mapping of LpMab-19 using deletion mutants of hPDPN in flow cytometry. Each hPDPN deletion mutant (dN23, dN37, dN46, dN55, dN64, dN75, dN85, dN95, and dN105) was treated with LpMab-19 (**A**, 1 μg/mL; red), PMab-1 (anti-MAP tag; **B**, 1 μg/mL; green), or control PBS (**A**, **B**; black) for 30 minutes at 4°C, followed by treatment with antimouse IgG or antirat IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800.
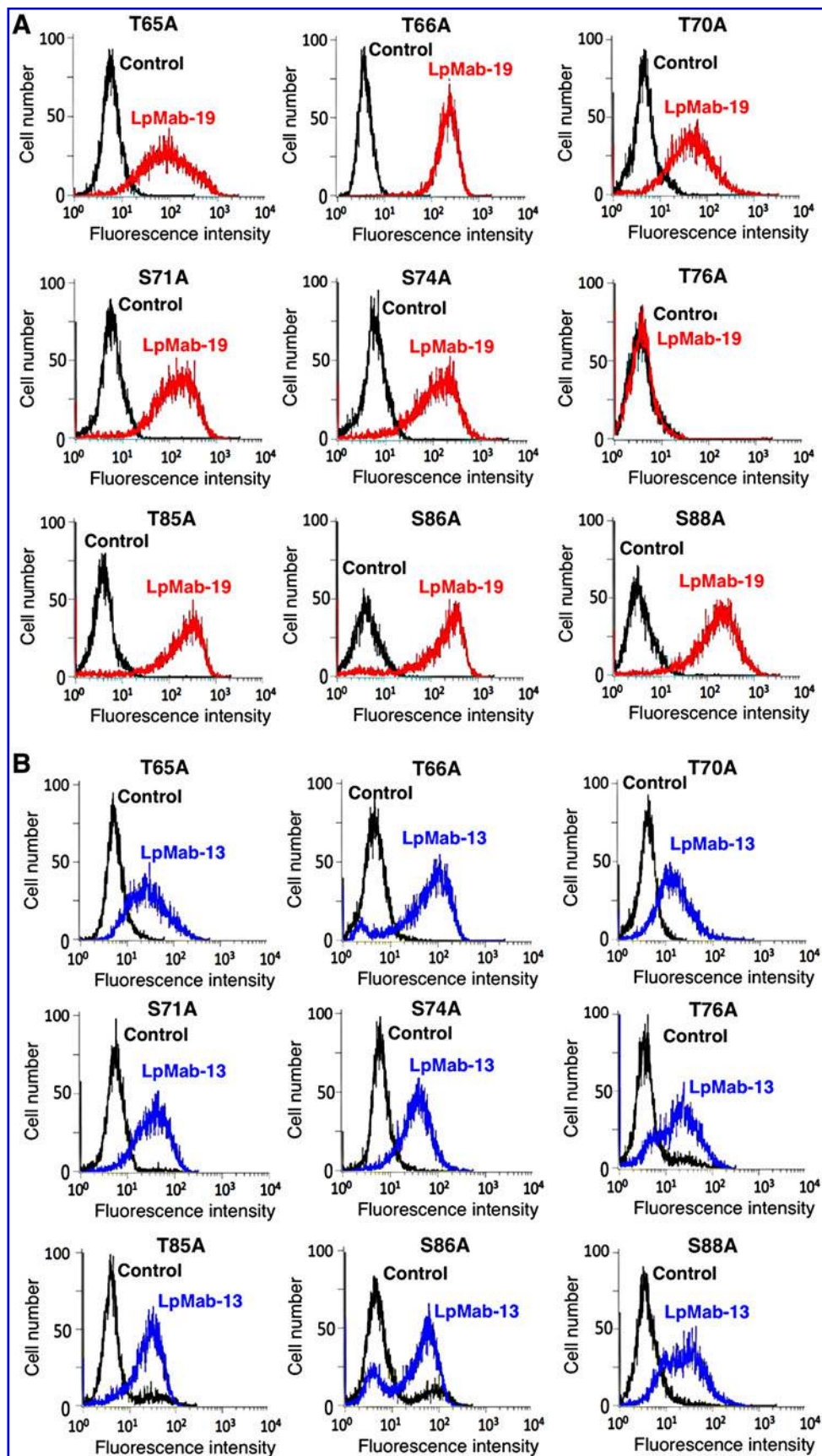
FIG. 3. Epitope mapping of LpMab-19 using point mutants of Ser/Thr residues of hPDPN in flow cytometry. Stable CHO-S transfectants expressing hPDPN point mutants (T65A, T66A, T70A, S71A, S74A, T76A, T85A, S86A, and S88A) were treated with LpMab-19 **(A**, 1 μg/mL; red), LpMab-13 **(B**, 1 μg/mL; blue), or control PBS **(A, B**; black) for 30 minutes at 4°C, followed by treatment with antimouse IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. CHO, Chinese hamster ovary.

Similarly, LpMab-19 detected dN37, dN46, dN55, dN64, and dN75. In contrast, LpMab-19 lost the reaction with dN85, dN95, and dN105, indicating that the N-terminus of the LpMab-19 epitope exists between the 75th and 85th amino acids (Fig. 2A). All deletion mutants of hPDPN possess the N-terminal MAP tag and were detected by the anti-MAP tag mAb (clone: PMab-1) (Fig. 2B).

Next, we produced stable CHO-S cell lines expressing point mutants of hPDPN: T65A, T66A, T70A, S71A, S74A, T76A, T85A, S86A, and S88A (Fig. 3A). The epitope of LpMab-19 was thought to include glycans because LpMab-19 did not react with synthetic peptides of hPDPN (data not shown).

LpMab-19 lost the reaction with the T76A mutant, whereas the other point mutants were detected by LpMab-19 (Fig. 3A). All point mutants targeting Ser/Thr residues were detected by LpMab-13 (Fig. 3B). Therefore, the epitope of LpMab-19 includes sialic acid on O-glycan, which is attached to Thr76.

We further determined the LpMab-19 epitope using alanine scanning around Thr76 of hPDPN. Nine hPDPN point mutants against Ser74–Thr85 were transiently expressed in CHO-K1 cells. As shown in Figure 4A, LpMab-19 did not detect T76A, I78A, and R79A. In contrast, LpMab-13 recognized all point mutants (Fig. 4B), indicating that the minimum epitope of LpMab-19 was Thr76–Arg79.
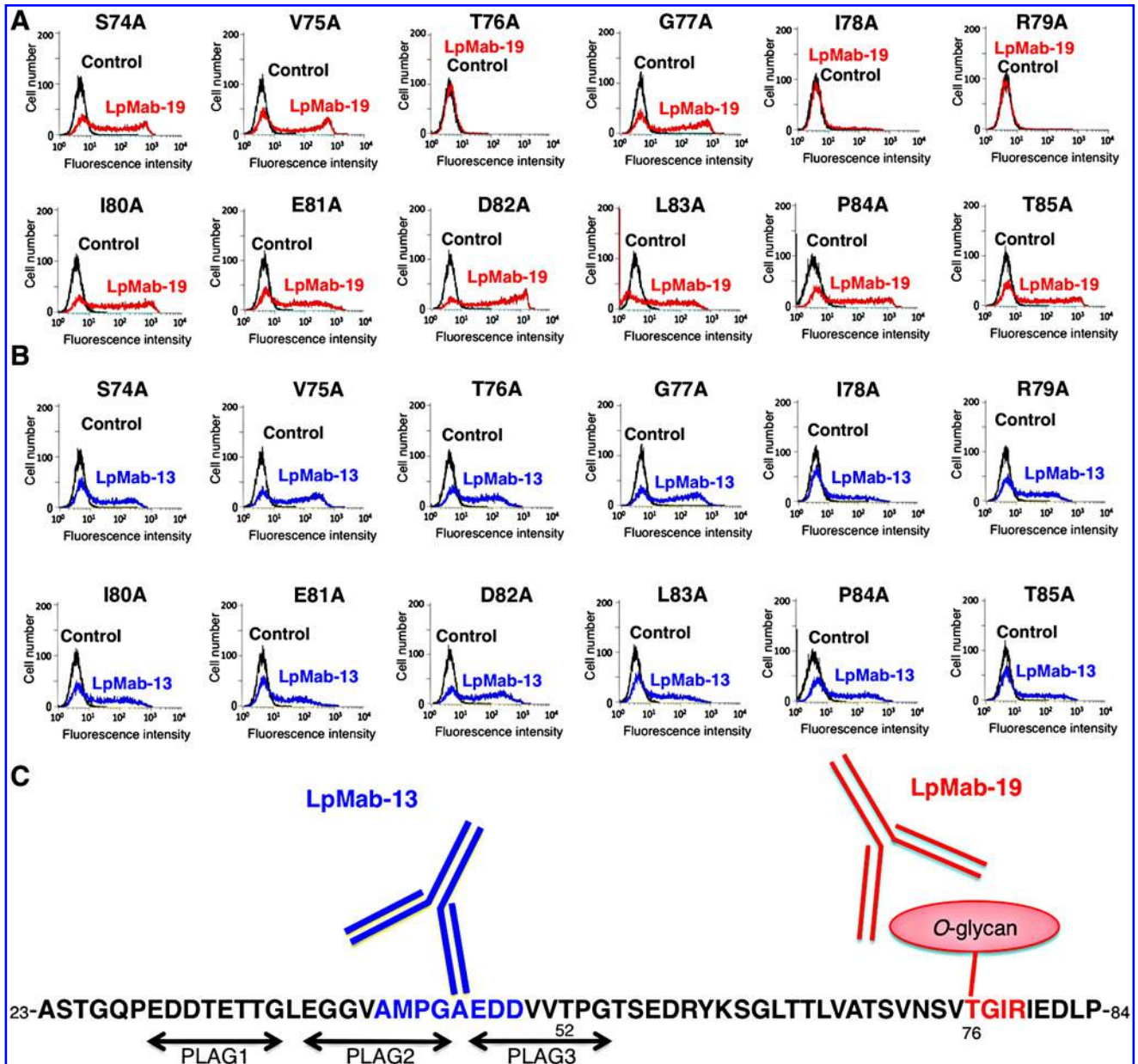

FIG. 4. Epitope mapping of LpMab-19 using point mutants of hPDPN in flow cytometry. Nine hPDPN point mutants against Ser74-Thr85 were transiently expressed in CHO-K1 cells. Cells were treated with LpMab-19 (**A**, 1 µg/mL; red), LpMab-13 (**B**, 1 µg/mL; blue), or control PBS (**A, B**; black) for 30 minutes at 4°C, followed by treatment with antimouse IgG-Oregon green. Fluorescence data were collected using a Cell Analyzer EC800. (**C**) Schematic illustration of the epitope of anti-hPDPN mAbs. mAbs, monoclonal antibodies.
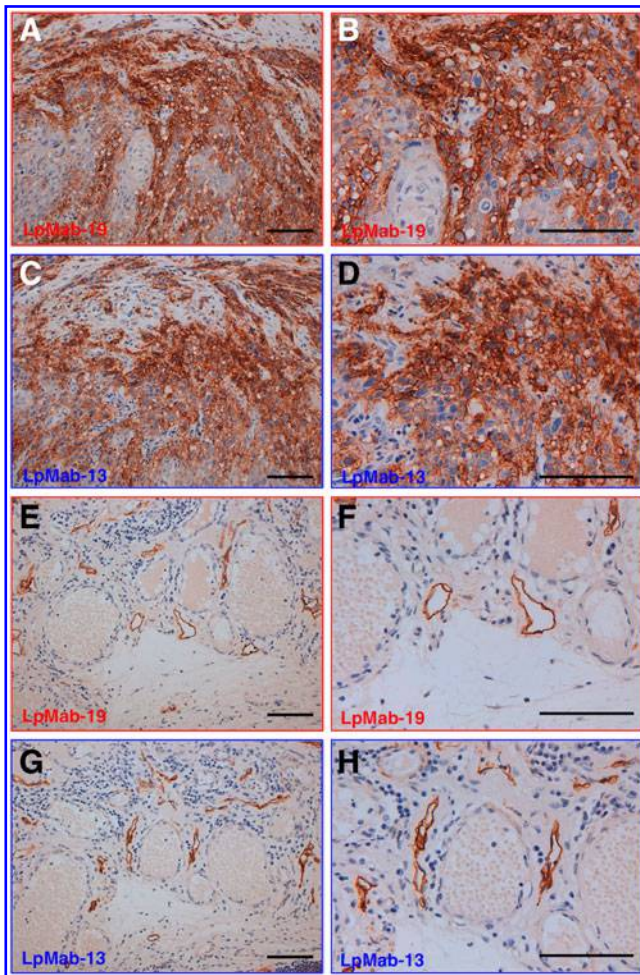
FIG. 5. Immunohistochemical analysis by LpMab-19 against oral squamous cell carcinoma. Sections were incubated with 1 μg/mL of LpMab-19 **(A, B, E, F)** or LpMab-13 **(C, D, G, H)**, followed by Envision+ kit, and color was developed using DAB and counterstained with hematoxylin. Scale bar: 100 μm. DAB, 3,3-diaminobenzidine tetrahydrochloride.

It has been reported that hPDPN is expressed in many cancers, such as malignant brain tumor, mesothelioma, oral cancer, lung cancer, esophageal cancer, testicular tumor, and osteosarcoma.[13,38,44] Here we compared the reactivity of LpMab-19 and LpMab-13 in an immunohistochemical analysis using oral squamous cell carcinoma. LpMab-19 stained cancer cells (Fig. 5A, B) and LECs (Fig. 5E, F) in a membrane/cytoplasmic-staining pattern without an antigen retrieval procedure. LpMab-19 did not react with vascular endothelial cells (Fig. 5E, F). The staining pattern of LpMab-19 is similar to LpMab-13, indicating that LpMab-19 is very useful for immunohistochemistry.

Therefore, LpMab-19 could be useful for investigating the expression and function of hPDPN in cancers and normal tissues. In future, different epitope-possessing anti-hPDPN mAbs should be produced for uncovering the function of hPDPN.

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Author Disclosure Statement

No competing financial interests exist.

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