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Establishment of a monoclonal antibody against glycosylated CD271 specific for cancer cells in immunohistochemistry

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Abstract

Various proteins are highly expressed in cancer (e.g., epidermal growth factor receptor); however, the majority are also expressed in normal cells, although they may differ in expression intensity. Recently, we reported that CD271 (nerve growth factor receptor), a glycosylated protein, increases malignant behavior of cancer, particularly stemlike phenotypes in squamous cell carcinoma (SCC). CD271 is expressed in SCC and in normal epithelial basal cells. Glycosylation alterations generally occur in cancer cells; therefore, we attempted to establish a cancer-specific anti-glycosylated CD271 antibody. We purified recombinant glycosylated CD271 protein, immunized mice with the protein, and screened hybridomas using an ELISA assay with cancer cell lines. We established a clone G4B1 against CD271 which is glycosylated with *O*-glycan and sialic acid. The G4B1 antibody reacted with the CD271 protein expressed in esophageal cancer, but not in normal esophageal basal cells. This specificity was confirmed in

Abbreviations: CIS, carcinoma in situ; CRD, cysteine-rich domain; FBS, fetal bovine serum; FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemistry; PBS, phosphate-buffered saline; RT, room temperature; SCC, squamous cell carcinoma.

hypopharyngeal and cervical cancers. G4B1 antibody recognized the fetal esophageal epithelium and Barrett's esophagus, which possess stem cell–like characteristics. In conclusion, G4B1 antibody could be useful for precise identification of dysplasia and cancer cells in SCC.

KEYWORDS
antibody, cancer stem cell, CD271, esophageal cancer, glycosylation

1 | INTRODUCTION

Squamous cell carcinoma (SCC) is one of the major cancer types, and there are few driver mutations and oncogenes because of the high complexity of genome alterations compared with adenocarcinoma. Cancer tissue is composed of different cancer cell types, and there are certain types of therapy-resistant cancer cells, known as cancer stem cells. We previously identified CD271 as a cancer stem cell marker in hypopharyngeal and lung SCC.[1,2] CD271, also known as p75NTR or NGFR, is a common receptor for all neurotrophins. We demonstrated that CD271-high cancer cells exhibit cisplatin resistance and high tumorigenicity, and loss of CD271 reduced cancer malignancy, including tumorigenicity, proliferation capacity, and migration activity. These data led us to hypothesize that CD271 could be a promising target for therapy in SCC. We previously established a humanized anti-CD271 antibody, which possesses antibody-dependent cellular cytotoxicity in vitro and in vivo. However, this approach is expected to affect both cancer cells and normal basal cells because CD271 is also expressed in epithelial basal cells; therefore, a more cancer-specific method is warranted.

Squamous cell carcinoma is a common esophageal cancer type in Japan, although adenocarcinoma in the setting of Barrett's esophagus is higher in the United States.[3] Intraepithelial neoplasia is used as an inclusive term for squamous dysplasia and carcinoma in situ (CIS), and SCC is generally considered to develop through dysplastic lesion progression or dysplasia-carcinoma sequence.[4]

Aberrant glycosylation is generally known to occur in cancer cells.[5] Numerous studies have demonstrated that the cancer-specific glycosylation pathway could play an essential role in cancer development,[6] but evidence of proteins with cancer-specific glycosylation is markedly limited. The antibody against podoplanin LpMab-2 can recognize esophageal cancer, but not normal lymphatic endothelial cells, both of which express normal or aberrantly glycosylated podoplanin.[7] The antibody against MUC21 heM21c, which binds to MUC21 with Tn, T, or sialyl-T epitopes, but not the unmodified core polypeptide of MUC21, recognizes esophageal cancer but not normal epithelium.[8] These data all suggested that esophageal cancer cells possessed several aberrantly glycosylated proteins not expressed in the normal epithelium.

CD271 has a single *N*-glycosylation site in the first cysteine-rich domain (CRD) and is rich in *O*-glycans, particularly in the juxtamembrane region.[9] An *O*-glycosylated stalk domain was reported to be required for apical sorting.[10] However, little is known about the type of glycosylation that occurs in CD271 in cancer cells and its biological and pathological significance.

Therefore, in this study, we established an antibody against glycosylated CD271 in cancer cells and analyzed the specificity of this antibody in pathology specimens.

2 | MATERIALS AND METHODS

2.1 | Ethics statements

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committees of the Miyagi Cancer Center (Natori, Japan). All procedures were approved by and executed in accordance with the Miyagi Cancer Center (permit number: 2019–081) and performed as per committee regulations.

2.2 | Cell lines

The human cell lines used were the hypopharyngeal patient-derived xenograft cell lines (HPCM1,[2,11] HPCM 2,[2,11] HPCM6[12] cells), which were maintained in RPMI-1640 medium (Wako) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin, and 100 μg/mL streptomycin. SK-MEL-2-Luc and MeWo-Luc cells (human malignant melanoma, maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin) were provided by JCRB. MCC148c cells[12] (human lung squamous carcinoma) were maintained in DMEM supplemented with 10% FBS, 0.4 mg/mL hydrocortisone, 2.5 mM Y-27632 (Focus Biomolecules), and penicillin/streptomycin. DMEM supplemented with 10% FBS and penicillin/streptomycin was used to maintain 293T cells (RIKEN BioResource Center). Chinese hamster ovary (CHO) cells and glycan-deficient CHO cell lines (Lec1, Lec2, and Lec8) were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI with 10% FBS.

2.3 | CD271 expression vector and its variants

The human CD271 expression vector pcDNA4/myc-HisA-hCD271WT[12] and CD271 deletion mutants (Δ1, Δ1-2, Δ1-3, and Δ1-4) have been described previously.[12]

2.4 | Establishment of genetically modified cell lines

Human CD271-overexpressing cells (SP2/0-hCD271, SK-OV-3-hCD271, and HPCM2-hCD271) were established using the retroviral vector pBABE-puro-CD271, as previously described.[12]

Human CD271-overexpressing CHO, Lec1, Lec2, and Lec8 cells were established as follows: the cell lines were transfected with pcDNA3.1-CD271 vector by electroporation, followed by adding G418 (0.5 mg/mL; Wako) and cell sorting (FACS AriaII, BD Biosciences).

2.5 | Purification of CD271 extracellular domain (ECD) recombinant protein

Purification of recombinant proteins was performed as previously described.[13] Briefly, *Escherichia coli* DH10Bac (Invitrogen) was transformed using the pFastBac-CD271 ECD recombinant donor plasmid. To produce recombinant baculovirus, the precultured Sf21 cells were transfected with recombinant bacmid DNA using the Cellfection II reagent (Invitrogen) according to the manufacturer's instructions. Lysis buffer (20 mM Tris-HCl, 10% glycerol, 1% NP-40, 1 mM EGTA, 1 mM DTT, 150 mM NaCl, pH8.0) with protease inhibitor cocktail (Sigma-Aldrich) was added to the collected cells, sonicated, and centrifuged. The supernatant was then rotated with Glutathione Sepharose 4 B (Sigma-Aldrich) for 60 minutes at 4 °C. PreScission Protease (GE Healthcare) was added to the mixture with PreScission Cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, pH7.5).

2.6 | Establishment of hybridoma cell lines

The establishment of hybridoma cell lines was performed as described previously.[12] The culture supernatant of hybridoma cells was screened by ELISA. To select the cancer-specific antibody, we performed the ELISA using hypopharyngeal cancer cell lines, HPCM2. HPCM2-hCD271 (human CD271-overexpressing cell) and HPCM2-EV (empty vector) cells were seeded in plates and fixed in 4% paraformaldehyde. Fixed cells were incubated with supernatant, HRP-conjugated anti-mouse IgG was applied, and the enzyme activity was detected using the substrate tetramethyl benzidine.

To determine the epitope, transiently transfected 293T cells (CD271 full length, Δ1, Δ1-2, Δ1-3, and Δ1-4) were seeded in polyethylenimine-coated plates and incubated for 24 hours.

To determine the antibody concentration, plates were coated with anti-mouse IgG2a IgG (ISO2-1 Kit; Sigma-Aldrich), washed, and incubated with the culture supernatant of hybridoma cells or anti-mouse IgG2a antibody as a standard (200 μg/mL, anti-CA IX, H-11, Santa Cruz Biotechnology).

The isotype of the antibody in the hybridoma supernatant was determined using mouse monoclonal antibody isotyping reagents (Sigma-Aldrich) following the manufacturer's instructions.

2.7 | Small interfering RNAs

Two CD271 siRNAs (siCD271-1 HSS107179 and siCD271-2 HSS107181) and nonsilencing control siRNA (siControl, 12935–300) were purchased from Invitrogen. siRNA transfections were performed using Lipofectamine RNAiMAX Reagent (Life Technologies) in an antibiotic-free medium for 48 hours. The siRNA knockdown efficiency was confirmed by flow cytometry analysis.

2.8 | Flow cytometry

Flow cytometry was performed as previously described.[11] Fluorescence data were collected using a FACSCanto II or SA3800 cell analyzer (BD Biosciences or Sony Biotechnology, respectively). Anti-CD271 antibody (ME20.4) was purchased from BioLegend. The data were analyzed using FlowJo (BD Biosciences) or CytoExploreR package on R software.[14]

2.9 | Immunocytochemistry

The cells were seeded in a glass-bottom dish (Matsunami). On the following day, the cells were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin for 15 minutes at room temperature (RT). After washing with PBS, the cells were subsequently incubated with Image-iT FX Signal Enhancer (Thermo Fisher Scientific) for 30 minutes at RT, washed with PBS, and incubated with the first antibody (G4B1, 50 μg/mL) for 1 hour at RT. After washing with PBS, the cells were further incubated with the secondary antibody (alexa488-conjugated anti-mouse IgG, 1:200, Thermo Fisher Scientific) and DAPI (1 μg/mL; Dojindo). The cells were observed using confocal microscopy (Nikon A1; Nikon).

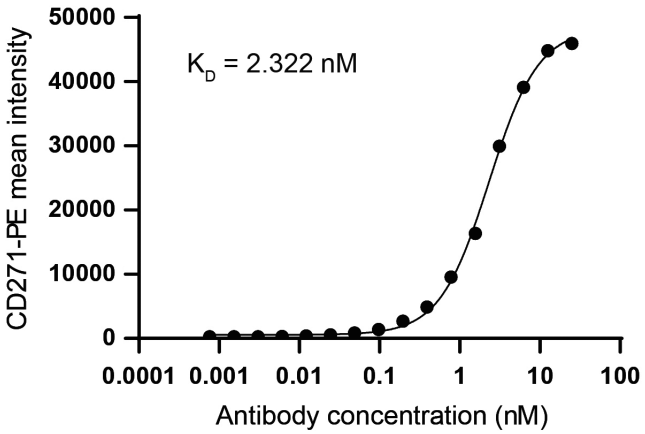

FIGURE 1 Binding affinity of the G4B1 antibody on SKOV3-CD271 cells. G4B1 and ME20.4 clones were used for ELISA assay. Fluorescence data were collected using flow cytometry, and the dissociation constant (K_D) values were calculated using GraphPad Prism8

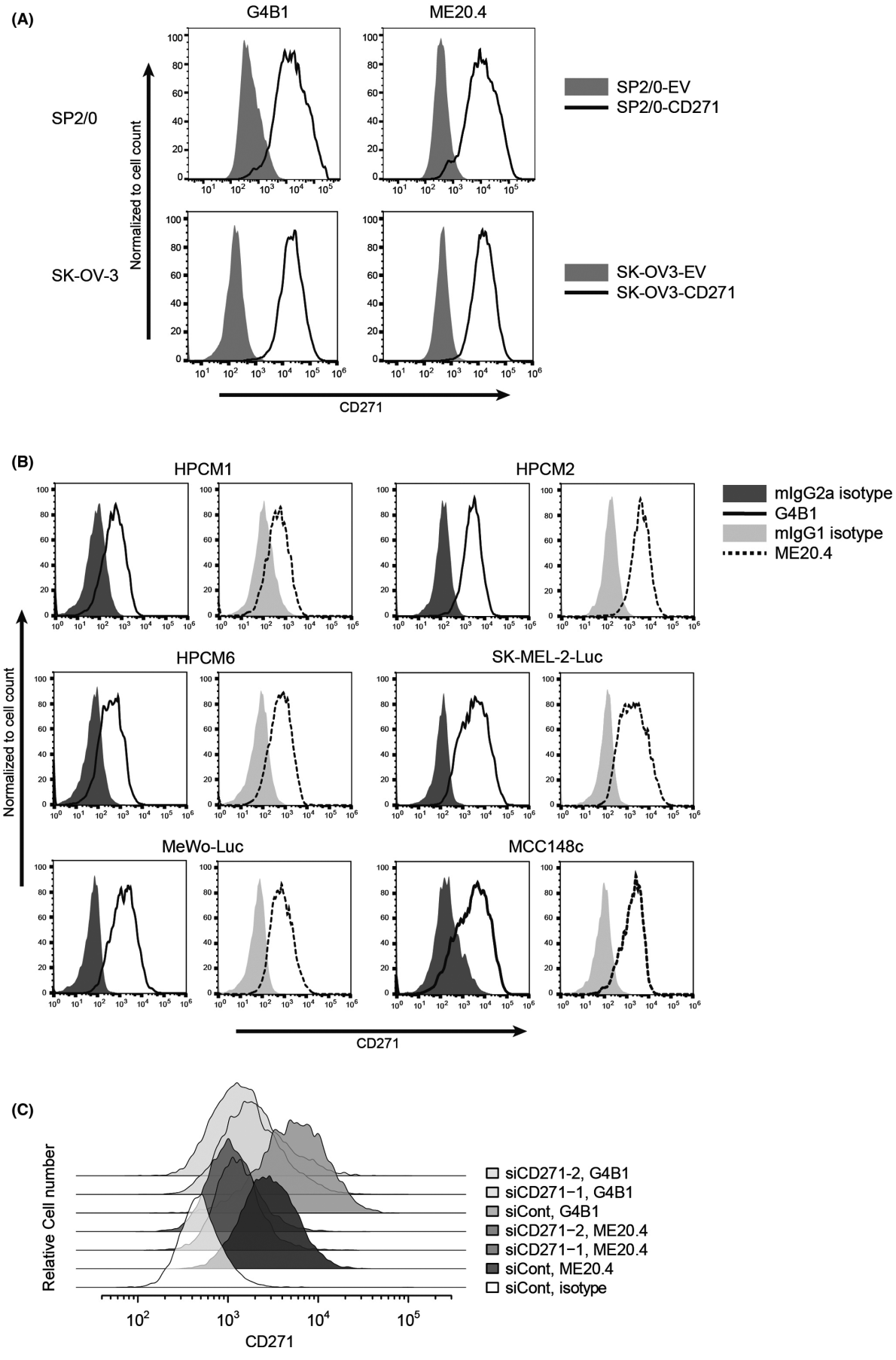
(A)
G4B1
ME20.4
SP2/0
SK-OV-3
Normalized to cell count
CD271
SP2/0-EV
SP2/0-CD271
SK-OV3-EV
SK-OV3-CD271
(B)
HPCM1
HPCM2
HPCM6
SK-MEL-2-Luc
MeWo-Luc
MCC148c
Normalized to cell count
CD271
mIgG2a isotype
G4B1
mIgG1 isotype
ME20.4
(C)
Relative Cell number
CD271
siCD271-2, G4B1
siCD271−1, G4B1
siCont, G4B1
siCD271−2, ME20.4
siCD271−1, ME20.4
siCont, ME20.4
siCont, isotype

FIGURE 2 G4B1 antibody specifically detects overexpressing and endogenous CD271. A, Flow cytometry analysis of G4B1 antibody using CD271-overexpressing SP2/0 and SK-OV-3 cells. EV, empty vector. B, Flow cytometry analysis of G4B1 and ME20.4 antibodies in the indicated cell lines. C, HPCM2 cells were knocked down by siRNA, and flow cytometry analysis was performed using G4B1 or ME20.4 antibody

2.10 | Immunohistochemistry (IHC)

An esophageal disease tissue array (ES2081) was purchased from Biomax. Barrett's esophagus and fetal esophagus (19-21 weeks) 10% formalin-fixed paraffin-embedded (FFPE) samples were obtained from Tohoku University Hospital. Hypopharyngeal and cervical disease FFPE samples were obtained from the Miyagi Cancer Center.

Immunostaining of human CD271 cells was performed as previously described.[11] Anti-CD271 antibody (C40-1457) was purchased from BD Biosciences. First antibody was diluted: G4B1, 1:9000; C40-1457, 1:2500. Anti-SOX2 antibody was purchased from Millipore (6F1.2, 1:400). Cell blocks were prepared as follows: a piece of paraffin was placed at the bottom of a 1.5-mL tube, to which cells were added. The tube was centrifuged, the supernatant was removed, and agar was added to the tube. The cells were fixed with 10% formaldehyde solution for 24 hours at RT. Next, the cell-containing blocks were processed in paraffin blocks.

2.11 | Western blotting

Western blotting was performed as previously described.[11] The antibodies used were as follows: anti-CD271 antibody (D4B3, Cell Signaling Technology, 1:1000; G323A, polyclonal antibody, Promega, 1:8000). To test nondenaturing CD271, cell lysates were boiled with sample buffer without 2-mercaptoethanol (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% sucrose) for 5 minutes. The samples were separated by SDS-PAGE gel (Bio-Rad) and transferred onto PVDF membranes (BioRad).

2.12 | Statistical analysis

For the evaluation of IHC, statistical tests were performed using the chi-square test with R software (version 4.1.0), and statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Production and characterization of novel anti-glycosylated CD271 monoclonal antibody clone G4B1

To develop a novel anti-CD271 mAb, we immunized mice with purified CD271ECD protein and prepared their splenic hybridoma cell culture. The culture supernatants were screened by ELISA for binding to hCD271-overexpressed HPCM2 cells. After limiting the

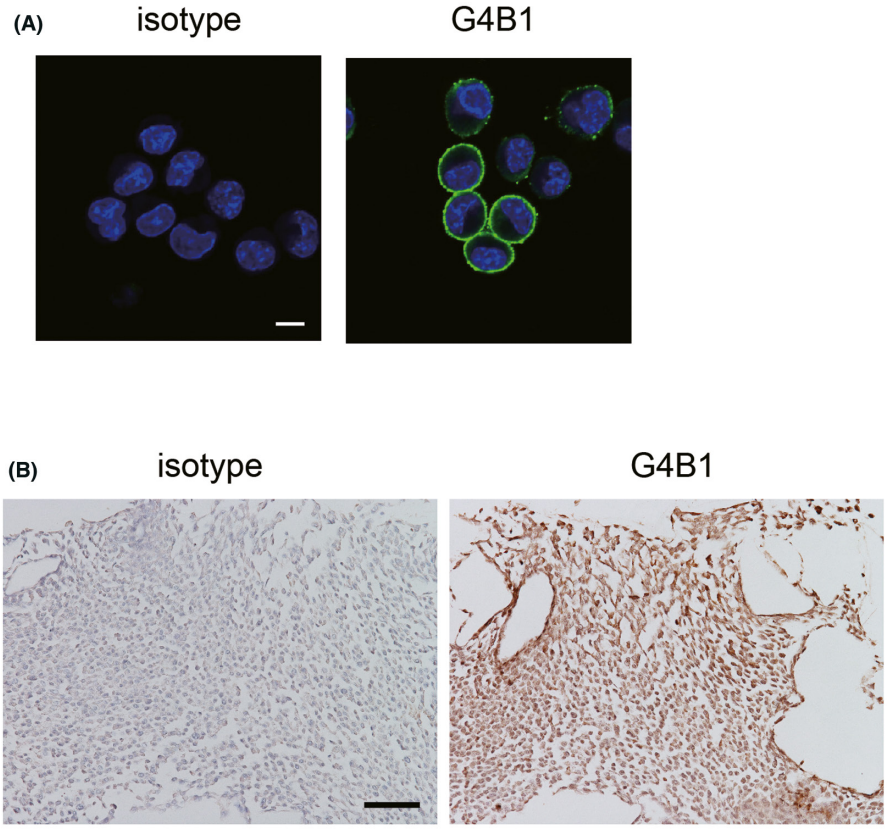

FIGURE 3 Immunocytochemistry using G4B1. A, Immunocytochemistry of empty vector- and human CD271-overexpressing SP2/0 cells using G4B1. Green, G4B1; blue, 4',6-diamidino-2-phenylindole. Bar: 10 μm. B, Immunohistochemistry of formalin-fixed paraffin-embedded SK-OV-3 cells expressing CD271 using G4B1. Bar: 100 μm

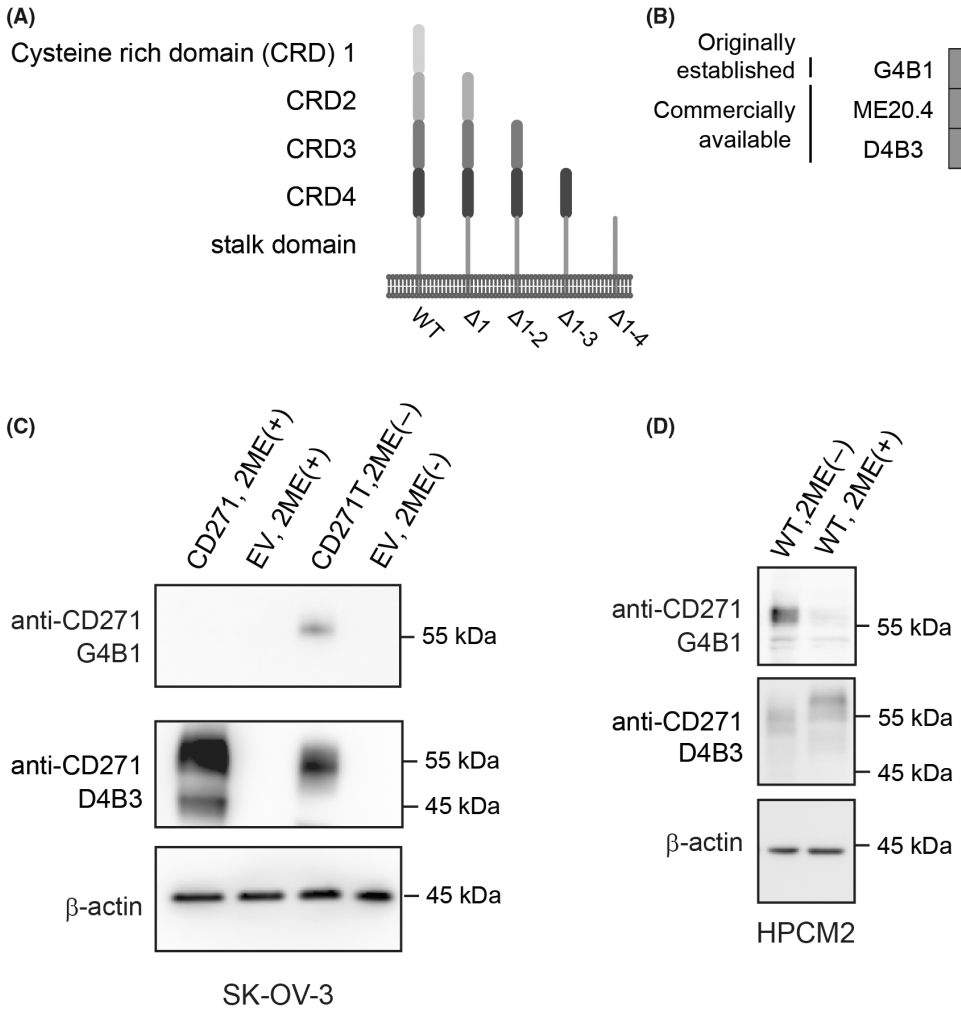

FIGURE 4 G4B1 recognizes conformational CRD4. A, A schema for the CD271 deletion mutant. B, Determination of the CD271 domain recognized by G4B1. The indicated human CD271 deletion mutants were transfected into 293T cells, and the affinity between the deletion mutants and the supernatant of G4B1 hybridoma cells was analyzed using ELISA. Values were normalized to the absorbance of hCD271 WT-expressing 293T cells. The boxes are shaded to indicate the values from minimum (white) to maximum (dark gray). C, Western blot analysis of SK-OV-3 control and CD271-overexpressing cells. EV, empty vector. 2ME, 2-mercaptoethanol in sample buffer. D, Western blot analysis of HPCM2 cells. WT, wild type. 2-ME, 2-mercaptoethanol in sample buffer [Correction added on 15 June 2022, after first online publication: in figure 4C, upper panel, the image was inverted vertically and molecular marker was corrected]

dilution of the hybridomas, clone G4B1 (IgG2a) was established. The dissociation curve of G4B1 was analyzed, and we found that G4B1 possessed higher affinity than clone ME20.4 (commercially available; Figure S1). We analyzed the kinetics of G4B1 in SK-OV3-CD271 cells using flow cytometry. The dissociation constant (K_D) of G4B1 was estimated to be 2.322 nM (Figure 1).

Next, we examined the affinity of G4B1 for human CD271 using cell lines by flow cytometry. When we used human CD271-overexpressing SP2/0 and SK-OV-3 cells, G4B1 clearly bound SP2/0-CD271 and SK-OV3-CD271 cells (Figure 2A). We checked the affinity of G4B1 for endogenous CD271-expressing human cancer cell lines. We found that the G4B1 and ME20.4 antibody bound endogenous CD271 (Figure 2B). To confirm the specificity of the G4B1 antibody against CD271, we examined its affinity to CD271-knockdown cells. The affinity of G4B1 to CD271-knockdown cells was clearly reduced in CD271-knockdown cells, which is similar to the affinity of the ME20.4 antibody (Figure 2C). Next, we tested whether the G4B1 antibody could be used for immunofluorescent staining. The G4B1 antibody recognized overexpressed CD271 in 293T cells (Figure 3A). We also found that the G4B1 antibody recognized FFPE SK-OV-3 cells overexpressing CD271 (Figure 3B), indicating that G4B1 antibody was useful for IHC in FFPE samples.

3.2 | Determination of the epitope

To determine the epitope for the G4B1 antibody, deletion mutants of CD271 were constructed (Figure 4A) and the mutants were transfected into 293T cells. We checked the affinity and found that the G4B1 antibody did not react with the deletion of CRD1 to the CRD4 mutant (Δ1-4; Figure 4B), suggesting that G4B1 binds to the CRD4 domain.

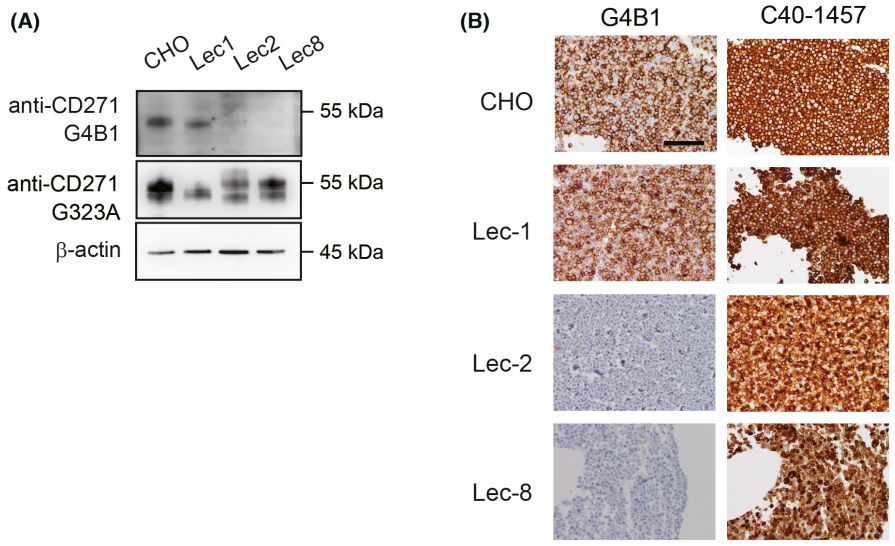

FIGURE 5 A, Western blot analysis of Chinese hamster ovary (CHO) and its derivative cell lines. G323A antibody was purchased from Promega. B, Immunohistochemistry (IHC) of CHO and its derivative cell lines. Cell blocks were prepared using indicated cell lines, and IHC was performed using G4B1 or BD antibodies (C40-1457). Bar: 100 μm

Next, we checked whether G4B1 could be used for Western blotting. We found that G4B1 antibody bound protein samples without 2-ME treatment, but not 2-ME–treated protein samples in CD271-overexpressing SK-OV-3 cells and CD271 endogenously expressing HPCM2 cells (Figure 4C and D), suggesting that G4B1 antibody could bind only to the conformational CD271 protein.

We generated this antibody using glycosylated CD271 antigen. Thus, we tested whether the G4B1 antibody is specific for certain glycosylation types. We established human CD271-expressing CHO cells that lack a specific glycosylation transferase (Lec1 lacks GlcNAc glycosyl transferase, resulting in N-linked carbohydrate deficiency; Lec2 lacks the CMP-sialic acid transporter, resulting in sialic acid deficiency; and Lec8 lacks the UDP-galactose transporter, resulting in *O*-glycan deficiency). In Western blotting, G4B1 antibody detected CD271 overexpression in wild-type CHO and Lec1 cells, but not in Lec2 and Lec8 cells (Figure 5A). We confirmed this specificity using IHC. CD271 protein was clearly observed in CHO and Lec1 cells, but not in Lec2 and Lec8 cells (Figure 5B), indicating that G4B1 can only detect CD271 with sialic acid and *O*-glycan modifications.

3.3 | Immunohistochemistry of esophageal diseases

We then studied the specificity of the G4B1 antibody using histopathological specimens. In normal esophageal tissue, basal cells were immunohistochemically positive for the C40-1457 antibody, while no positive cells were detected using the G4B1 antibody (Figure 6A). There was no immunoreactivity in the intraepithelial esophageal neoplasms and CIS with G4B1 antibody, but positivity was detected when using C40-1457. In SCC, both G4B1 and C40-1457 antibodies could detect positive carcinoma cells. We studied 26 cases of normal esophagus, and no immunoreactivity was detected in normal epithelium using G4B1 antibody, while 20 cases were positive using the C40-1457 antibody (Table 1).

3.4 | G4B1 binds fetal esophageal epithelium and Barrett's esophagus

We previously reported that CD271 played a pivotal role in cancer stem cells. Therefore, in this study, we hypothesized that the different glycosylation patterns between CD271 in normal epithelium and cancer could depend on the stemness of the cells examined. In order to further test this interesting hypothesis, we immunolocalized the fetal esophageal epithelium. The C40-1457 antibody could not detect any fetal esophageal cells, but the G4B1 antibody clearly bound to these fetal esophageal epithelial cells (Figure 6B). These cells were also immunohistochemically positive for SOX2, confirming that the fetal esophagus possesses stemlike characteristics.

We then tested the immunolocalization of CD271 in the esophageal adenocarcinoma arising in Barrett's esophagitis. We previously reported that CD271 was expressed specifically in SCC but not in adenocarcinoma.[1] In this study, G4B1 antibody yielded immunopositivity in carcinoma cells of Barrett's adenocarcinoma. However, the C40-1457 antibody marginally yielded positivity in adenocarcinoma (Figure 6C). G4B1 also yielded positivity in Barrett's epithelial cells undergoing intestinal metaplasia (Figure 6D). Both adenocarcinoma and metaplasia were immunohistochemically positive for SOX2, suggesting that these cells also possess a stemlike capacity.

3.5 | Immunolocalization in hypopharyngeal and cervical cancers

We further examined the specificity of the G4B1 antibody in hypopharyngeal and cervical cancers. In hypopharyngeal cancer (Figure 7A), C40-1457 antibody yielded positivity in the basal cells of normal and dysplastic lesion and cancerous lesion. However, the G4B1 antibody yielded no positivity in normal epithelium, dysplasia, and CIS, while it yielded marked positivity in carcinoma cells, which is consistent with the results of IHC in esophageal mucosa in our present study.

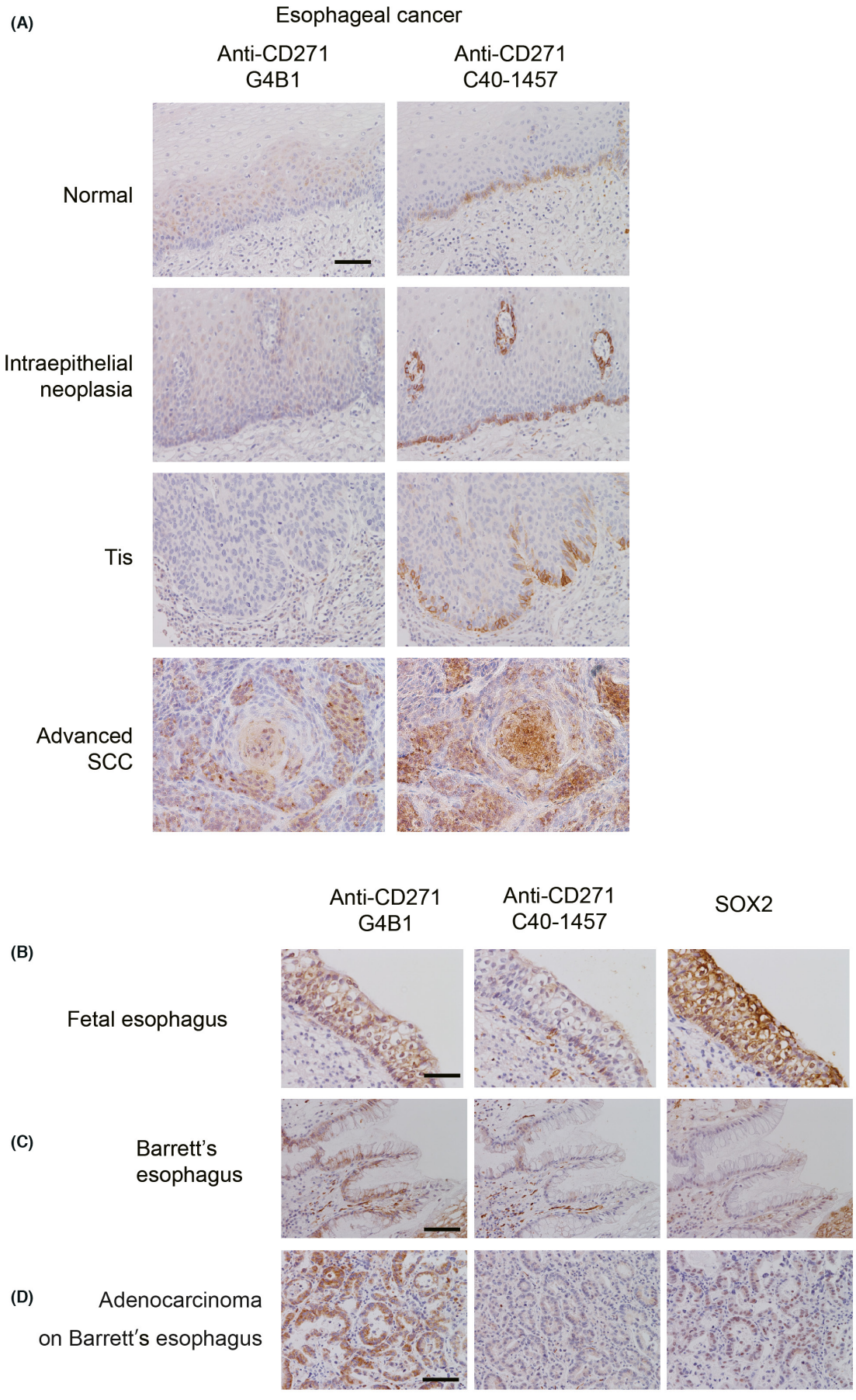

FIGURE 6 A, Immunohistochemistry of normal esophagus and esophageal cancer. Immunostaining was performed with anti-CD271 (G4B1 and C40-1457). SCC, squamous cell carcinoma. Bar, 100 μm. B-D, Immunohistochemistry of fetal esophagus (B), Barrett's esophagus (C), and adenocarcinoma (D). Immunostaining was performed with anti-CD271 (G4B1 and C40-1457) and anti-SOX2. Bar: 100 μm

TABLE 1 Number of positive cases using each anti-CD271 antibody in immunohistochemistry

	G4B1	C40-1457	*P* value
Normal ($n = 26$)	0	20	<0.001
IEN ($n = 18$)	0	15	<0.001
Tis ($n = 16$)	2	12	0.001
Advanced SCC ($n = 54$)	16	8	0.0699

Abbreviations: IEN, intraepithelial neoplasia; SCC, squamous cell carcinoma.

In the cervical normal epithelium (Figure 7B), positive cells were detected in the suprabasal area, but not in basal cells, using G4B1 antibody. Using C40-1457, positive cells were detected in the basal layer. In dysplasia or CIS, the staining area increased from the suprabasal to the middle area in stratified squamous epithelium and approximately all regions in CIS. In SCC, cancerous regions were positive in using both G4B1 and C40-1457 antibodies, which is also compatible with esophageal specimens.

4 | DISCUSSION

We demonstrated that the recognition site of CD271 by the G4B1 antibody is the CRD4 domain. We also revealed that the G4B1 antibody was specific to the conformational epitope of CRD4 and glycosylated CD271. The G4B1 antibody recognizes cancerous regions, but not in the normal epithelium, both of which express the CD271 protein. These data suggested that specific glycosylation, including CD271, was specifically increased through esophageal oncogenesis.

The G4B1 antibody recognized overexpressing CD271 protein in CHO and 293T (human embryonic kidney) cells, whose origins are not human cancer. Previous studies demonstrated that an antibody against cancer-specific glycosylated podoplanin recognized overexpressing human podoplanin in CHO cells.[7,15] Therefore, overexpressing CD271 in CHO cells could be glycosylated similar to the cancer cells.

In this study, we found that the G4B1 antibody could bind to Barrett's epithelium and adenocarcinoma. We previously reported that CD271 was not expressed in adenocarcinomas. A previous report demonstrated that the expression of SOX2 and OCT3, both of which are essential for maintaining pluripotency, was elevated in Barrett's esophagus and esophageal adenocarcinoma as compared with normal epithelium.[16] In a mouse model, Lgr5+ gastric stem cells were reported to be present in Barrett's esophagus developed by bile acid exposure.[17] These data all suggested that Barrett's esophagus and adenocarcinoma possessed stemlike characteristics. We also demonstrated that the G4B1 antibody recognized the fetal esophagus. These data indicated that the G4B1 antibody could recognize esophageal epithelial cells harboring stemlike features by IHC.

In cervical cancer, G4B1 antibody did not react with the normal basal layer, but the staining was positive in the suprabasal layer of dysplasia/CIS, which is different from esophageal and hypopharyngeal cancer. The staining intensity in the suprabasal layer gradually increased from normal to CIS, suggesting that the G4B1 antibody could recognize not only cancer cells but also early atypical cells. Further studies are required to elucidate the role of aberrant glycosylated CD271 in premalignant cervical lesions.

In this study, we demonstrated that the G4B1 antibody recognized esophageal carcinoma cells but not non-neoplastic epithelial

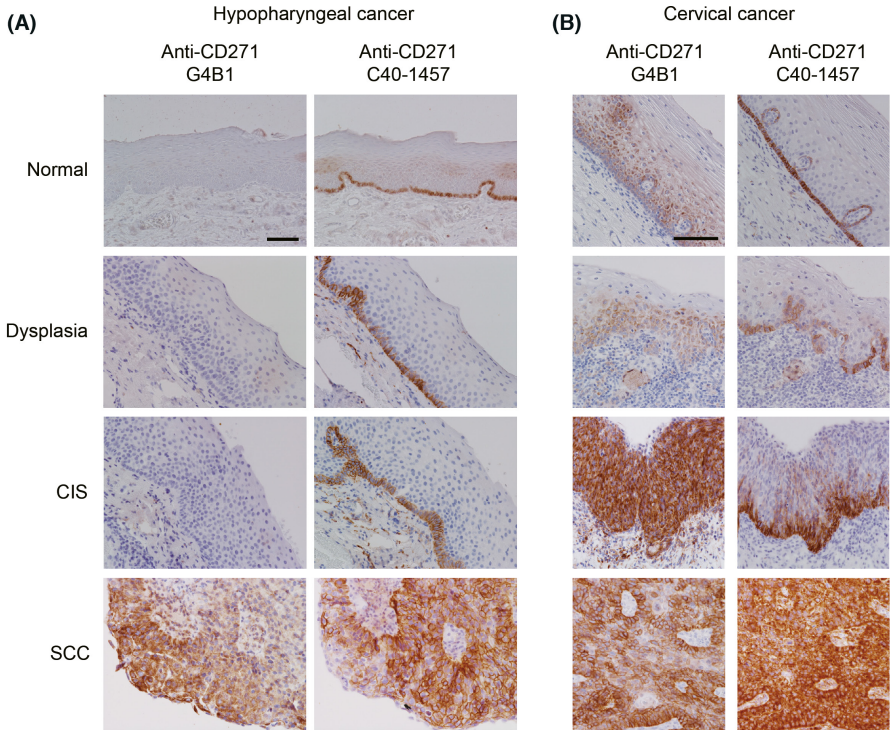

FIGURE 7 Immunohistochemistry of hypopharyngeal (A) and cervical cancer (B). Immunostaining was performed with anti-CD271 (G4B1 and C40-1457). CIS, carcinoma in situ; SCC, squamous cell carcinoma. Bar: 100 μm

cells. This antibody can be useful as a prominent marker that clearly distinguishes between normal and malignant epithelium by IHC. Future analysis of aberrant glycosylated CD271 will shed light on the mechanisms of oncogenesis in the esophagus.

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DISCLOSURE

The authors have no conflict of interest to disclose.

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REFERENCES

1. Mochizuki M, Nakamura M, Sibuya R, et al. CD271 is a negative prognostic factor and essential for cell proliferation in lung squamous cell carcinoma. *Lab Invest*. 2019;99(9):1349-1362.
2. Imai T, Tamai K, Oizumi S, et al. CD271 defines a stem cell-like population in hypopharyngeal cancer. *PLoS One*. 2013;8(4):e62002.
3. Shimizu M, Nagata K, Yamaguchi H, Kita H. Squamous intraepithelial neoplasia of the esophagus: past, present, and future. *J Gastroenterol*. 2009;44(2):103-112.
4. Taylor PR, Abnet CC, Dawsey SM. Squamous dysplasia–the precursor lesion for esophageal squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2013;22(4):540-552.
5. Stowell SR, Ju T, Cummings RD. Protein glycosylation in cancer. *Annu Rev Pathol*. 2015;10:473-510.
6. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer*. 2015;15(9):540-555.
7. Kato Y, Kaneko MK. A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. *Sci Rep*. 2014;4:5924.
8. Tian Y, Denda-Nagai K, Kamata-Sakurai M, et al. Mucin 21 in esophageal squamous epithelia and carcinomas: analysis with glycoform-specific monoclonal antibodies. *Glycobiology*. 2012;22(9):1218-1226.
9. Johnson D, Lanahan A, Buck CR, et al. Expression and structure of the human NGF receptor. *Cell*. 1986;47(4):545-554.
10. Yeaman C, Le Gall AH, Baldwin AN, Monlauzeur L, Le Bivic A, Rodriguez-Boulan E. The O-glycosylated stalk domain is required for apical sorting of neurotrophin receptors in polarized MDCK cells. *J Cell Biol*. 1997;139(4):929-940.
11. Mochizuki M, Tamai K, Imai T, et al. CD271 regulates the proliferation and motility of hypopharyngeal cancer cells. *Sci Rep*. 2016;6:30707.
12. Morita S, Mochizuki M, Wada K, et al. Humanized anti-CD271 monoclonal antibody exerts an anti-tumor effect by depleting cancer stem cells. *Cancer Lett*. 2019;461:144-152.
13. Morita S, Mochizuki M, Shibuya-Takahashi R, et al. Establishment of a monoclonal antibody that recognizes cysteine-rich domain 1 of human CD271. *Monoclon Antib Immunodiagn Immunother*. 2020;39(1):6-11.
14. Hammill D. CytoExploreR: Interactive Analysis of Cytometry Data [Internet]. 2020. Available from: https://github.com/DillonHammill/CytoExploreR
15. Kaneko MK, Nakamura T, Kunita A, et al. ChLpMab-23: Cancer-specific human-mouse chimeric anti-podoplanin antibody exhibits antitumor activity via antibody-dependent cellular cytotoxicity. *Monoclon Antib Immunodiagn Immunother*. 2017;36(3):104-112.
16. Wang X, Yang S, Zhao X, et al. OCT3 and SOX2 promote the transformation of Barrett's esophagus to adenocarcinoma by regulating the formation of tumor stem cells. *Oncol Rep*. 2014;31(4):1745-1753.
17. Quante M, Bhagat G, Abrams JA, et al. Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett-like metaplasia. *Cancer Cell*. 2012;21(1):36-51.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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