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Article

Development of a novel anti-CD44 monoclonal antibody C44Mab-46 for multiple applications against esophageal squamous cell carcinomas

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Abstract: CD44 is a cell surface glycoprotein, which is widely expressed on normal and cancer cells. CD44 is involved in cell adhesion, migration, proliferation, survival, stemness, and chemo-resistance. Therefore, CD44 is thought to be a promising target for cancer diagnosis and therapy. In this study, we established anti-CD44 monoclonal antibodies (mAbs) by immunizing mice with CD44v3-10 ectodomain and screening using enzyme-linked immunosorbent assay. We then characterized them using flow cytometry, western blotting, and immunohistochemistry. One of the established clones (C44Mab-46; IgG_1, kappa) reacted with CD44s-overexpressed Chinese hamster ovary-K1 cells (CHO/CD44s) or esophageal squamous cell carcinoma (ESCC) cell lines (KYSE70 and KYSE770). The K_D of C44Mab-46 for CHO/CD44s, KYSE70, and KYSE770 was 1.1×10^{-8} M, 4.9×10^{-8} M, and 4.1×10^{-8} M, respectively. C44Mab-46 detected CD44s of CHO/CD44s and KYSE70, and CD44v of KYSE770 in western blot analysis. Furthermore, C44Mab-46 strongly stained esophageal squamous carcinoma cells in immunohistochemistry using formalin-fixed paraffin-embedded ESCC tissues. Taken together, C44Mab-46 is very useful for detecting CD44 in various applications.

Keywords: CD44; monoclonal antibody; esophageal cancer

1. Introduction

CD44 is a cell surface glycoprotein, which is widely expressed on various tissues [1]. The CD44 gene is comprised of 19 exons in human [2]. The first five and the last five exons are constant and encode the shortest isoform of CD44 (85–95 kDa), called as CD44 standard isoforms (CD44s). The middle nine exons can be alternatively spliced and assembled with the ten exons contained in CD44s. They are referred to as CD44 variant isoforms (CD44v) [3]. CD44 is a major receptor for hyaluronic acid (HA), and is involved in cell adhesion, migration, and proliferation [4]. CD44 also plays important roles in tumor progression, metastasis, and resistance to chemo- and radiotherapy [5,6]. Therefore, CD44 is a promising target for cancer diagnosis and therapy.

Several CD44-targeting monoclonal antibodies (mAbs) have been developed for preclinical researches. IM7 mAb inhibited HA-induced VEGF production in human vascular endothelial cells [7]. IM7 mAb significantly decreased cell migration and invasion in breast cancer cells [8]. In pancreatic cancer, H4C4 mAb reduced tumor growth, metastasis, and post-radiation recurrence [9]. A humanized mAb specific for CD44, RG7356, was directly cytotoxic for leukemia B cells, but no effect on normal B cells. Administration of RG7356 to immune-deficient mice engrafted with human chronic lymphocytic leukemia cells resulted in complete clearance of engrafted leukemia cells [10]. Furthermore, CD44v6-specific humanized mAbs (BIWA-4 and BIWA-8) labeled with ^{186}Re showed the therapeutic efficacy in head and neck SCC xenograft bearing nude mice [11].

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Sulfasalazine is an inhibitor of xCT, the subunit of cystine-glutamate transporter. CD44v was found to interact with the xCT and promotes oxidative stress resistant in cancer cells [12,13]. The clinical trials of sulfasalazine have been evaluated in combination with cisplatin in patients with CD44v-expressing advanced gastric cancer refractory to cisplatin [14], and the reduction of the CD44v-positive gastric cancer cells [15]. These results suggest that CD44v is an important marker to predict and evaluate the sensitivity of sulfasalazine.

Previously, we established the novel mAb, C$_{44}$Mab-5 (IgG$_1$, kappa) against CD44 using Cell-Based Immunization and Screening (CBIS) method [16]. C$_{44}$Mab-5 showed high sensitivity for flow cytometry and immunohistochemical analysis in oral cancers. In this study, we developed a novel anti-CD44 mAb, C$_{44}$Mab-46 (IgG$_1$, kappa), and evaluated it for various applications, including flow cytometry, western blotting, and immunohistochemical analyses.

2. Materials and Methods

2.1. Cell lines

Mouse multiple myeloma P3X63Ag8U.1 (P3U1), a glioblastoma cell line (LN229) [17], and CHO-K1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Esophageal squamous cell carcinoma (ESCC) cell lines, KYSE70 and KYSE770 were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan).

CD44s ORF was amplified from LN229 cDNA using HotStar HiFidelity Polymerase Kit (Qiagen Inc., Hilden, Germany). CD44s ORF was subcloned into pCAG-Ble-ssPA16 vector possessing signal sequence and N-terminal PA16 tag (GLEGGVAMPGAEDDVV) [16,18-22]. CHO/CD44s was established by transfecting pCAG-Ble/PA16-CD44s into CHO-K1 cells using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). CD44v3-10 ectodomain (CD44ec)-secreting LN229 (LN229/CD44ec) was established by transfecting pCAG-Neo/PA-CD44ec-RAP-MAP into LN229 cells using the Neon transfection system. The amino acid sequences of the tag system in this study were as follows: PA tag [23-25], 12 amino acids (GVAMPGAEDDVV); RAP tag [26,27], 12 amino acids (DMVNPGLEDRIE); and MAP tag [28,29], 12 amino acids (GDGMVPPGIEDK).

LN229, KYSE70, and KYSE770 were cultured in Dulbecco's Modified Eagle Medium (DMEM) complete medium, including DMEM (4.5 g/L glucose) with L-Gln and without sodium pyruvate (Nacalai Tesque, Inc., Kyoto, Japan), 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific Inc.), 100 U/mL of penicillin (Nacalai Tesque, Inc.), 100 μg/mL streptomycin (Nacalai Tesque, Inc.), 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). LN229/CD44ec was cultured in DMEM complete medium, including 0.5 mg/ml of G418 (Nacalai Tesque, Inc.).

P3U1 and CHO-K1 were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc.) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. CHO/CD44s was cultured in RPMI complete medium, including 0.5 mg/mL Zeocin (InvivoGen, San Diego, CA, USA). All cells were grown in a humidified incubator at 37°C with 5% CO_2.

2.2. Purification of CD44ec

After LN229/CD44ec was cultured using DMEM complete medium without G418, CD44ec was purified from the supernatants using RAP tag system, comprised an anti-RAP tag mAb (clone PMab-2) and a RAP peptide (GDDMVNPGLEDRIE) [26,27]. The filtered culture supernatant (5L) was passed through PMab-2-Sepharose (2 mL bed volume), and the same process was repeated three times. The beads were then washed with 100 mL of phosphate-buffered saline (PBS, Nacalai Tesque, Inc.), and eluted with 0.1 mg/mL a RAP peptide in a step-wise manner (2 mL × 10).

2.3. Hybridoma production

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Female BALB/c mice (6-week-old) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under specific pathogen-free conditions. The Animal Care and Use Committee of Tohoku University approved all animal experiments.

A BALB/c mouse was immunized with CD44ec (150 μg) intraperitoneally (i.p.) with Imject Alum (Thermo Fisher Scientific Inc.). The procedure included three additional immunizations with CD44ec (2nd immunization, 150 μg; 3rd immunization, 100 μg; 4th immunization, 50 μg) followed by a final booster injection of CD44ec (50 μg) two days prior to the harvest of splenic cells.

Subsequently, splenic cells were fused with P3U1 cells using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN, USA). The hybridomas were then grown in RPMI media supplemented with hypoxanthine, aminopterin, and thymidine (HAT) for selection (Thermo Fisher Scientific Inc.). The culture supernatants were screened for the anti-CD44s antibody production using enzyme-linked immunosorbent assay (ELISA).

2.4. ELISA

CD44ec was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc) at a concentration of 1 μg/mL for 30 min at 37℃. After washing with PBS containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc.), wells were blocked with 1% bovine serum albumin (BSA)-containing PBST for 30 min at 37℃. Culture supernatants were added to each well, followed by peroxidase-conjugated anti-mouse immunoglobulins (1:2000 diluted; Agilent Technologies Inc., Santa Clara, CA, USA). Enzymatic reactions were conducted, using 1 Step Ultra TMB (Thermo Fisher Scientific Inc.), followed by the measurement of the optical density at 655 nm, using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

2.5. Flow cytometry

Cells were collected following a brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). The cells were then washed with 0.1% BSA in PBS and treated with C_{44}Mab-46 for 30 min at 4°C. After incubation, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA, USA). Fluorescence data were collected using SA3800 Cell Analyzer (Sony Corp.) and analyzed using FlowJo (BD Biosciences, Franklin Lakes, NJ, USA).

2.6. Determination of dissociation constant (K_D) through flow cytometry

CHO/CD44s, KYSE70, and KYSE770 cells were suspended in 100 μL serially-diluted anti-C_{44}Mab-46 mAbs, after which 50 μL Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.) was added. Afterward, fluorescence data were collected, using BD FACSLyric. The K_D was subsequently calculated by fitting saturation binding curves to the built-in; one-site binding models in GraphPad PRISM 8 (GraphPad Software, Inc., La Jolla, CA, USA).

2.7. Western blot analysis

Cell lysates (10 μg) were boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.). Proteins were separated on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.) in PBS with 0.05% Tween 20, membranes were incubated with 1 μg/mL C_{44}Mab-46 or 1 μg/mL anti-β-actin (clone AC-15; Sigma-Aldrich Corp., St. Louis, MO, USA). Membranes were then incubated with peroxidase-conjugated anti-mouse immunoglobulins (diluted 1:1000; Agilent Technologies, Inc.) to detect C_{44}Mab-46 and anti-β-actin. Finally, protein bands were detected with a chemiluminescence reagent, ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

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2.8. Immunohistochemical analysis

Paraffin-embedded ESCC tissue microarray (Product Code: BC02011, US Biomax Inc.) were deparaffinized in xylene and rehydrated. Then, they were autoclaved in citrate buffer (pH 6.0; Agilent Technologies Inc.) for 20 min. After blocking with SuperBlock T20 (Thermo Fisher Scientific, Inc.), sections were incubated with C$_{44}$Mab-46 (5 μg/mL) for 1h at room temperature and then treated with the EnVision+ Kit for mouse (Agilent Technologies Inc.) for 30 min. Color was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies Inc.) for 2 min. Counterstaining was performed with hematoxylin (FUJIFILM Wako Pure Chemical Corporation). Hematoxylin & eosin (HE) staining (FUJIFILM Wako Pure Chemical Corporation) was performed using consecutive tissue sections. Leica DMD108 (Leica Microsystems GmbH, Wetzlar, Germany) was used to examine the sections and obtain images.

3. Results

3.1. Flow cytometric analysis of C$_{44}$Mab-46 to CD44 expressing cells

In this study, C$_{44}$Mab-46 was established by immunizing one mouse with CD44v3-10 ectodomain (Fig. 1). We have already confirmed the epitope of C$_{44}$Mab-46 as $_{174}$-TDDDV-$_{178}$ [30,31] encoded within the first five exons, indicating that C$_{44}$Mab-46 recognizes CD44 standard isoforms (CD44s). Therefore, we confirmed the reactivity of C$_{44}$Mab-46 against CHO/CD44s by flow cytometry. As shown in Fig. 2A, C$_{44}$Mab-46 recognized CHO/CD44s cells in a dose dependent manner, but not CHO-K1 cells (Fig. 2B). We next examined whether C$_{44}$Mab-46 could recognize endogenous CD44 in ESCC cell lines. C$_{44}$Mab-46 reacted with both KYSE 70 and KYSE770 in a dose dependent manner (Fig. 2C and 2D).

Next, we assessed the binding affinity of C$_{44}$Mab-46 with CHO/CD44s, KYSE70, and KYSE770 using flow cytometry. The K_D of C$_{44}$Mab-46 for CHO/CD44s, KYSE70, and KYSE770 was 1.1×10^{-8} M, 4.9×10^{-8} M, and 4.1×10^{-8} M, respectively, indicating that C$_{44}$Mab-46 possesses moderate affinity for CD44s-expressing cells (Fig. 3).

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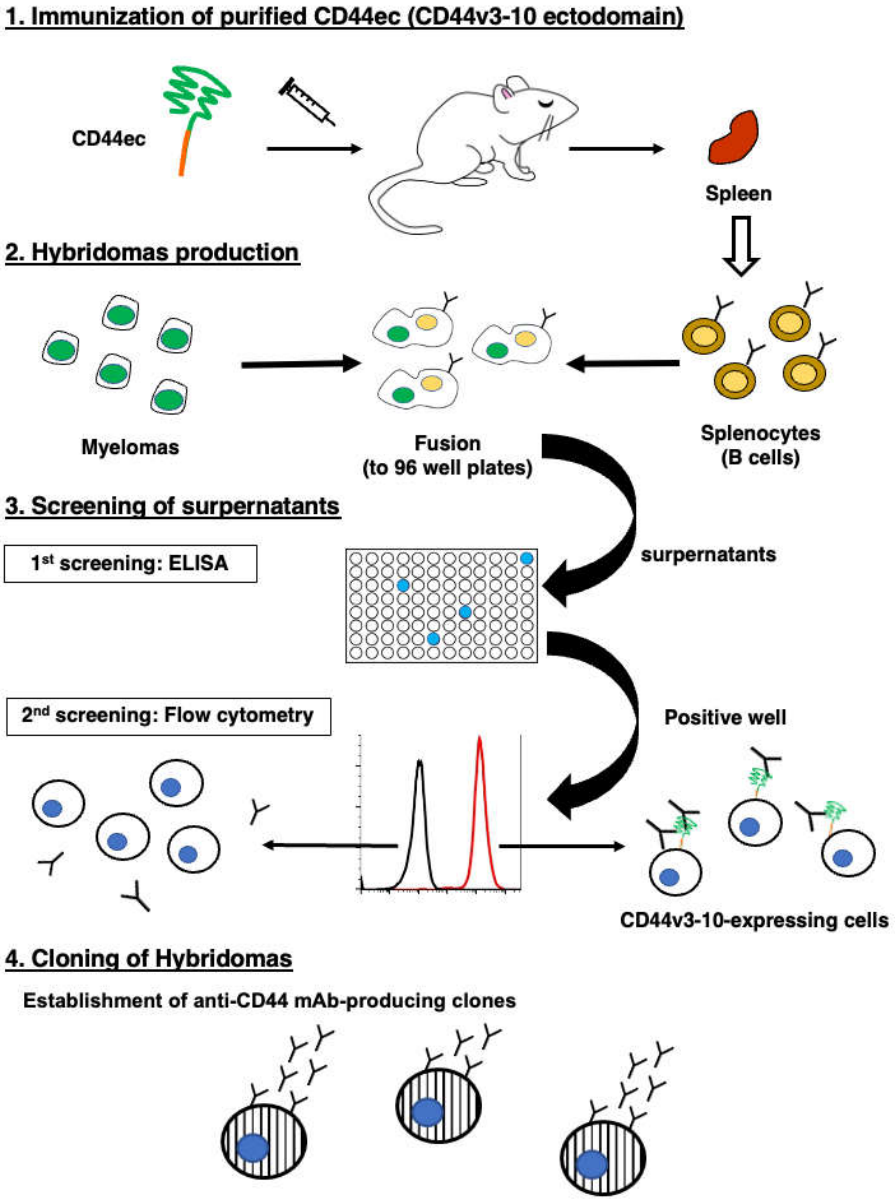

Figure 1. A schematic procedure of anti-CD44 mAbs production. Mice were intraperitoneally immunized with the purified CD44v3-10 ectodomain. The screening was then conducted by ELISA using the purified CD44v3-10 ectodomain, flow cytometry using parental cells, and CD44v3-10-overexpressed CHO-K1 cells.

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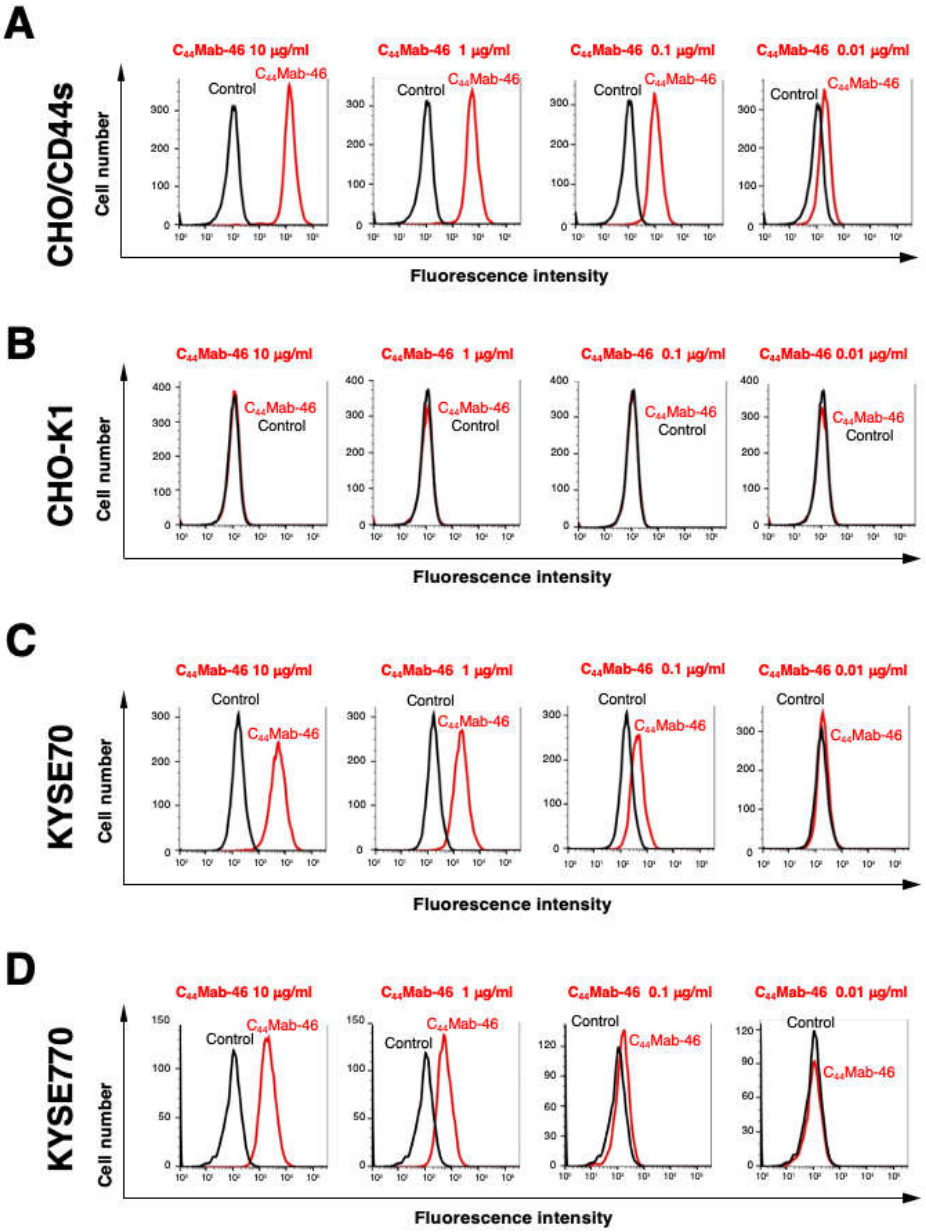

Figure 2. Flow cytometry to CD44 expressing cells using C44Mab-46. CHO/CD44s (A), CHO-K1 (B), KYSE70 (C) and KYSE770 (D) cells were treated with 0.01–10 μg/mL of C44Mab-46, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Black line represents the negative control.

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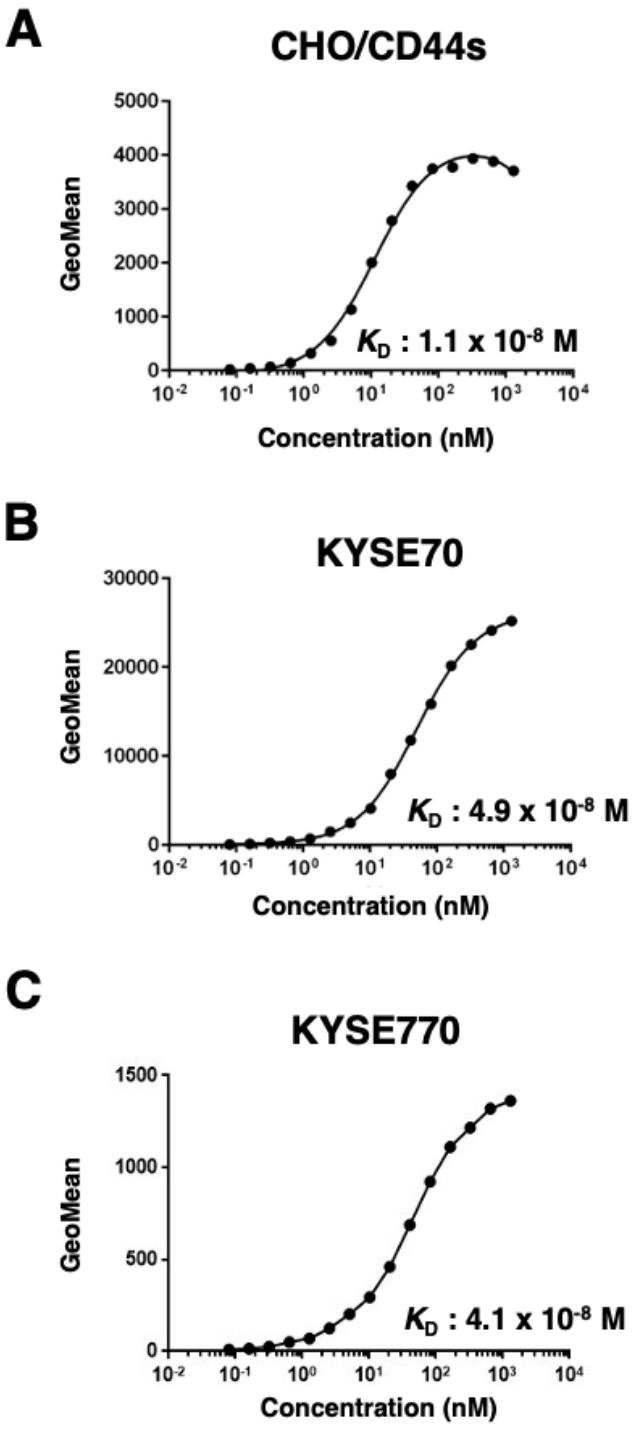

Figure 3. The determination of the binding affinity of C$_{44}$Mab-46. CHO/CD44s (A), KYSE70 (B), and KYSE770 (C) cells were suspended in 100 μL serially diluted C$_{44}$Mab-46 (6 ng/mL to 100 μg/mL). Then, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were subsequently collected using a BD FACSLyric, following the calculation of the dissociation constant (K_D) by GraphPad PRISM 8.

3.2. Western blot analysis

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Western blotting was performed to further assess the sensitivity of C44Mab-46. Lysates of CHO-K1 and CHO/CD44s cells were probed. As shown in Fig. 4A, C44Mab-46 detected CD44s as a ~85kDa band. However, C44Mab-46 did not detect any band from lysates of CHO-K1 cells. These results indicated that C44Mab-46 specifically detect exogenous CD44s.

Next, we examined the detection of endogenous CD44 using lysates from KYSE70 and KYSE770 cells. As shown in Fig. 4B, C44Mab-46 could detect CD44 as 85 and 48kDa double bands from lysates of KYSE70 cells. In contrast, C44Mab-46 could detect CD44 as more than 100 kDa triplet bands, probably CD44v isoforms, from lysates of KYSE770 cells. These results suggest that C44Mab-46 recognizes endogenous CD44s and CD44v(s) in the esophageal cancer cell lines.

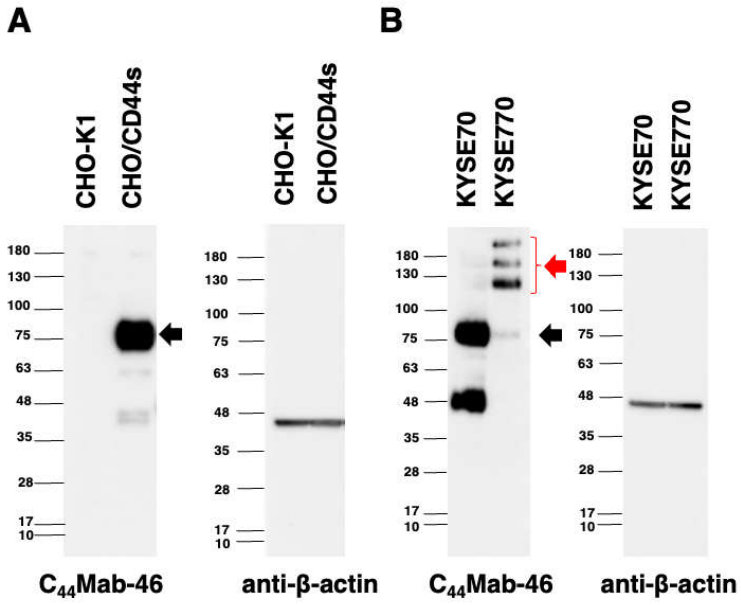

Figure 4. Western blotting by C44Mab-46. (A) Cell lysates of CHO-K1 and CHO CD44s (10 μg) were electrophoresed and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with 1 μg/mL of C44Mab-46 and 1 μg/mL of anti-β-actin and subsequently with peroxidase-conjugated anti-mouse immunoglobulins. (B) Cell lysates of KYSE70 and KYSE770 (10 μg) were electrophoresed and transferred onto PVDF membranes. The membranes were incubated with 1 μg/mL of C44Mab-46 and 1 μg/mL of anti-β-actin and subsequently with peroxidase-conjugated anti-mouse immunoglobulins. Black arrows indicate the predicted size of CD44s (~85 kDa). Red arrow indicates the CD44 variants.

3.3. Immunohistochemical analysis using C44Mab-46 against ESCC tissues.

To investigate whether C44Mab-46 can be used for immunohistochemical analyses using paraffin-embedded tumor sections, we used tissue microarray of ESCC. In a well differentiated ESCC section (Fig. 5A~D), C44Mab-46 strongly stained ESCC cells (Fig. 5A). A clear membrane-staining in ESCC was observed (Fig. 5B). Hematoxylin and eosin (HE) staining was performed using the serial sections (Fig. 5C and 5D). In an ESCC section with stromal invaded phenotype (Fig. 5E~H), C44Mab-46 strongly stained stromal invaded ESCC (Fig. 5E and 5F). HE staining was also performed using the serial sections (Fig. 5G and 5H). In immunohistochemical analyses using C44Mab-46 against ESCC tissue microarrays (Table 1), C44Mab-46 stained 63 of 67 (94.0%) cases of ESCC. These results indicated that C44Mab-46 is useful for immunohistochemical analysis of paraffin-embedded tumor sections.

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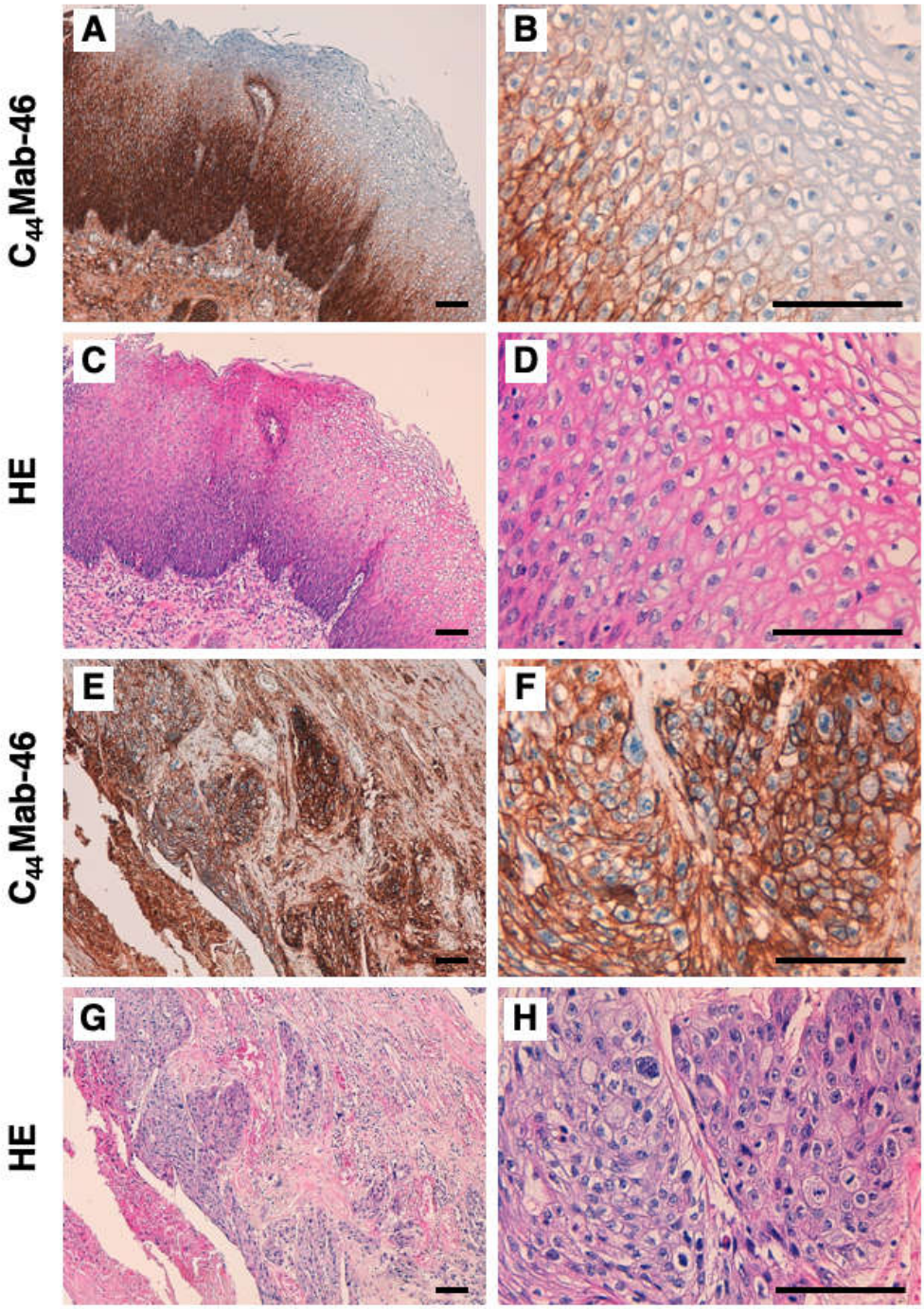

Figure 5. Immunohistochemical analysis using C44Mab-46 against ESCC tissues. (A, B, E, F) After antigen retrieval, sections were incubated with 5 μg/mL of C44Mab-46 followed by treatment with the Envision+ kit. Color was developed using DAB for 2min, and sections were counterstained with hematoxylin. (C, D, G, H) Hematoxylin and eosin (HE) staining. Scale bar = 100 μm.

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Table 1 Immunohistochemical analysis using C44Mab-46 against ESCC tissues

Case No.		Age	Sex	Organ/Anatomic Site	Pathology diagnosis	Grade	Type	Intensity
1		52	M	Esophagus	Squamous cell carcinoma	I	malignant	++
2		62	F	Esophagus	Squamous cell carcinoma	I	malignant	++
3		54	M	Esophagus	Squamous cell carcinoma	I	malignant	++
4		59	M	Esophagus	Squamous cell carcinoma	I	malignant	+
5		60	M	Esophagus	Squamous cell carcinoma	II	malignant	++
6		66	M	Esophagus	Squamous cell carcinoma	-	malignant	+
7		36	M	Esophagus	Squamous cell carcinoma	I	malignant	++
8		55	F	Esophagus	Squamous cell carcinoma	I	malignant	-
9		59	F	Esophagus	Squamous cell carcinoma	I-II	malignant	++
10		48	M	Esophagus	Squamous cell carcinoma	I-II	malignant	++
11		41	M	Esophagus	Squamous cell carcinoma	I	malignant	++
12		58	M	Esophagus	Squamous cell carcinoma	I	malignant	++
13		56	M	Esophagus	Squamous cell carcinoma	I	malignant	++
14	(Fig. 5E-H)	72	F	Esophagus	Squamous cell carcinoma	I	malignant	+++
15		41	M	Esophagus	Squamous cell carcinoma	I-II	malignant	+++
16		50	M	Esophagus	Squamous cell carcinoma	I	malignant	-
17		48	M	Esophagus	Squamous cell carcinoma	I	malignant	+
18		55	M	Esophagus	Squamous cell carcinoma	I-II	malignant	-
19		61	F	Esophagus	Squamous cell carcinoma	II	malignant	+
20		35	M	Esophagus	Squamous cell carcinoma	I	malignant	++
21		72	M	Esophagus	Squamous cell carcinoma	I	malignant	++
22		70	M	Esophagus	Squamous cell carcinoma	-	malignant	+++
23		42	F	Esophagus	Squamous cell carcinoma	I	malignant	++
24		53	M	Esophagus	Squamous cell carcinoma	I	malignant	++
25		54	M	Esophagus	Squamous cell carcinoma	I-II	malignant	++
26	(Fig. 5A-D)	54	F	Esophagus	Squamous cell carcinoma	I-II	malignant	+++
27		65	F	Esophagus	Squamous cell carcinoma	I-II	malignant	++
28		63	F	Esophagus	Squamous cell carcinoma	I-II	malignant	+++
29		62	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
30		63	M	Esophagus	Squamous cell carcinoma	II	malignant	++
31		65	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
32		64	F	Esophagus	Squamous cell carcinoma	II	malignant	++
33		71	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
34		55	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
35		57	F	Esophagus	Squamous cell carcinoma	II	malignant	+++
36		56	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
37		60	M	Esophagus	Squamous cell carcinoma	II-III	malignant	+++
38		61	F	Esophagus	Squamous cell carcinoma	II	malignant	++
39		61	M	Esophagus	Squamous cell carcinoma	II-III	malignant	+++
40		50	M	Esophagus	Smooth muscle and fatty tissue	-	malignant	+++
41		66	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
42		45	M	Esophagus	Squamous cell carcinoma	II	malignant	++
43		68	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
44		58	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
45		57	M	Esophagus	Squamous cell carcinoma	II-III	malignant	++
46		54	F	Esophagus	Squamous cell carcinoma	II	malignant	+
47		48	M	Esophagus	Squamous cell carcinoma	II	malignant	++
48		68	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
49		54	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
50		70	M	Esophagus	Squamous cell carcinoma	III	malignant	+++
51		72	M	Esophagus	Squamous cell carcinoma	III	malignant	++
52		62	M	Esophagus	Squamous cell carcinoma	III	malignant	-
53		63	M	Esophagus	Squamous cell carcinoma	II-III	malignant	++
54		49	F	Esophagus	Squamous cell carcinoma	II-III	malignant	++
55		53	F	Esophagus	Squamous cell carcinoma	II-III	malignant	++
56		61	M	Esophagus	Squamous cell carcinoma	III	malignant	+++
57		61	M	Esophagus	Squamous cell carcinoma	II	malignant	+++
58		59	F	Esophagus	Squamous cell carcinoma	III	malignant	+++
59		62	M	Esophagus	Squamous cell carcinoma	II-III	malignant	+++
60		56	M	Esophagus	Squamous cell carcinoma	III	malignant	+++
61		73	F	Esophagus	Squamous cell carcinoma	II-III	malignant	+++
62		57	M	Esophagus	Squamous cell carcinoma	II-III	malignant	++
63		64	M	Esophagus	Squamous cell carcinoma	III	malignant	+++
64		60	M	Esophagus	Squamous cell carcinoma	II-III	malignant	+++
65		66	M	Esophagus	Squamous cell carcinoma	III	malignant	+++
66		67	M	Esophagus	Squamous cell carcinoma	II-III	malignant	+++
67		75	M	Esophagus	Squamous cell carcinoma	III	malignant	+++

4. Discussion

CD44 have functions in many processes in normal cells (hematopoietic, immune system, and organogenesis), and in pathological situations (inflammation and cancer) [32]. CD44 exhibits passive ligand binding including adhesion to hyaluronan and other components of the extracellular matrix. Furthermore, CD44 acts as a co-receptor for growth factors and as a scaffold for enzymes. Therefore, establishment and characterization of

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anti-CD44 mAbs are thought to be important for development of CD44 targeting therapy and diagnosis.

In this study, we developed C44Mab-46 which can recognize all isoforms of CD44, and show the usefulness for flow cytometry (Fig. 2 and Fig. 3), western blotting (Fig. 4), and immunohistochemistry (Fig. 5). As shown in Fig.2, C44Mab-46 similarly recognized endogenous CD44 of KYSE70 and KYSE770 cells by flow cytometry; however, the expression pattern of CD44s and CD44v was different in western blot analysis (Fig. 4). Furthermore, we found the 48-kDa band in KYSE70 cells. By the radio labelled CD44s pulse chasing assay, a CD44s precursor form (about 48 kDa) was first translated, after which the precursor received *N*- and *O*-glycosylation and reached 85kDa mature form [33]. This result suggests that the 48-kDa band is a non-glycosylated precursor form of CD44 in KYSE70 cells. We previously determined the epitope of C44Mab-46 as 174-TDDDV-178 by enzyme-linked immunosorbent assay using synthetic peptides [31]. Although the Thr174 of CD44 have been confirmed as an *O*-glycan site [34], C44Mab-46 could recognize the epitope in the absence of glycosylation. In the future, it should be determined whether the 48-kDa CD44 is exposed on cell surface, and *O*-glycosylation at Thr174 affect the recognition by C44Mab-46.

CD44s and CD44v interact with various proliferation-, migration-, and invasion-promoting membrane proteins. CD44s binds to platelet-derived growth factor β-receptor and transforming growth factor-β type I receptor [35], and modulate their functions. CD44s also interacts with podoplanin (PDPN) and colocalize at cell-surface protrusions. PDPN-induced migration requires CD44 in MDCK cells, and knockdown of CD44 and PDPN in oral SCC cells affect cell spreading, suggesting that CD44 directly interacts PDPN with and modulates the intracellular signaling and cell migration [36]. It is interesting whether C44Mab-46 inhibits these protein-protein interactions and influence their functions.

CD44v6 is highly expressed in many cancers, and act as co-receptor for at least three receptor tyrosine kinases (c-Met [37,38] , EGFR [39], and VEGFR-2 [40]), which contribute to the oncogenic functions of CD44v6. Therefore, humanized anti-CD44v6 mAb BIWA4 (bivatuzumab)-mertansine drug conjugate, was evaluated in clinical trials [41]. However, the clinical trials were discontinued because of the severe skin toxicities [42,43]. A first-in-human phase I clinical trial with RG7356, targeting to the constant region of CD44, showed an acceptable safety profile in patients with advanced CD44-expressing solid tumors. However, the study was terminated due to no evidence of a clinical and/or pharmacodynamic dose-response relationship with RG7356, but not due to safety concerns [44]. Therefore, the development of anti-CD44 targeting mAbs with more potent and fewer side effects is desired.

We previously established cancer-specific mAbs (CasMabs) targeting to PDPN [45-48] and podocalyxin [49], which are expressed on many cancers including ESCC [50-53]. It is worthwhile to develop a cancer-specific anti-CD44 antibody using CasMab method. We previously converted the IgG_1 subclass of C44Mab-5 into a mouse IgG_{2a}, named 5-mG_{2a}, and produced a defucosylated version, 5-mG_{2a}-f. The 5-mG_{2a}-f exhibited ADCC/ CDC activities *in vitro* significantly reduced tumor growth in oral cancer cells [54]. Therefore, the production of a class switched and defucosylated version of C44Mab-46 is warranted to evaluate the *in vivo* anti-tumor activity. Since KYSE70, used in this study, was reported to be tumorigenic in athymic nude mice [55], the cell line can be used to evaluate the anti-tumor activity as *in vivo* model.

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