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Development of a Novel Anti-HER2 Monoclonal Antibody H_2Mab-181 for Gastric Cancer

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Human epidermal growth factor receptor 2 (HER2) is a type I transmembrane 185 kDa protein. HER2 is expressed in a variety of normal tissue types and cancer cells. HER2 is associated with cell proliferation, differentiation, and migration. The overexpression of HER2 has been observed in a number of cancers, including breast and gastric cancers. Gastric cancer is one of the most common cancers worldwide, with an annual case rate of ~1 million people diagnosed with the disease. Trastuzumab is a humanized anti-HER2 monoclonal antibody (mAb) that has been utilized in gastric cancer therapy. In this study, we have developed a novel anti-HER2 mAb, H_2Mab-181 (IgG_1, kappa), through the immunization of mice with a purified recombinant extracellular domain of HER2. H_2Mab-181 can specifically and sensitively detect HER2 in both flow cytometry and Western blot applications in gastric cancer cell lines and can also be utilized in immunohistochemical analyses of gastric cancer tissues. Together, H_2Mab-181 could be useful for the diagnosis and therapy in gastric cancers.

Keywords: HER2, monoclonal antibody, gastric cancer

Introduction

Human epidermal growth factor receptor 2 (HER2)/ErbB2/Neu is a 185 kDa type I transmembrane glycoprotein that is composed of an extracellular region, a single transmembrane region, and an intracellular region.[1] The extracellular region consists of four domains named I, II, III, and IV.[2] Among the HER family, which consists of EGFR, HER2, HER3, and HER4, the HER2 receptor ligand has not been identified and is represented as an activated structure without ligand binding.[3] Homodimers or heterodimers are formed between HER2 and other HER family members,[4] and these complexes promote signaling cascades that affect cell proliferation, differentiation, and migration.[5] Dimers that include HER2 have a higher tyrosine kinase activity, and among them, HER2/HER3 heterodimer has the greatest transforming and mitogenic signaling ability.[6,7]

HER2 is expressed in a variety of cell types, excluding the hematopoietic origin.[8] Overexpression and mutation of HER2 have been reported in many cancers, including breast, ovarian,[9] lung,[10] colorectal,[11] and gastric cancers.[12] Recently, drugs that specifically target HER2, such as trastuzumab and pertuzumab, have been widely used. Trastuzumab is a humanized murine monoclonal antibody (mAb) and was the first approved anti-HER2 agent for breast cancer in 1998.[13] Pertuzumab, which is a fully-humanized mAb that targets a different site (domain II) to trastuzumab (domain IV), was approved for breast cancer combination therapy with trastuzumab and docetaxel in 2012.[14] The use of trastuzumab in gastric cancers began in 2010.[15]

Gastric cancer is the fifth most common newly diagnosed cancer and is the fourth leading cause of death according to Global Cancer Statistics 2020.[16] Incidence rates are particularly high in East Asia and Eastern European regions. As the initial symptoms are clinically silent or asymptomatic, gastric cancer is often diagnosed at an advanced stage.[17] Although the 5-year survival rate is 80% for those patients with stage I, the survival rate is less than 20% in those

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diagnosed with stage IV.[18] Improvement to lifestyle habits and early diagnosis have resulted in a decrease in the number of cases and mortality[19]; however, incidence rates have started to increase recently within the young population.[20] Treatment for gastric cancer involves resection alone for early-stage disease, and chemotherapy with or without radiotherapy is also included for those diagnosed at an advanced stage.[21] Chemotherapy involves combinations of platinum-based drugs, where fluoropyrimidine and anthracycline are commonly used.[15] For those tumors that are unresectable or recurrent cases with HER2-positive gastric cancers, trastuzumab-containing regimens are recommended.[21] HER2 expression occurs in 10%–20% of gastric cancer cases, and some studies have reported that overexpression of HER2 is associated with poor outcomes.[12] The use of trastuzumab improved the prognosis of these patients; however, resistance often occurs in a relatively short time (median PFS 6.7 months).[22] The combination use of pertuzumab is currently undergoing clinical trials[23,24]; however, Tabernero *et al.* reported that combining pertuzumab and trastuzumab with chemotherapy did not significantly improve overall survival in a phase 3 study.[24] Therefore, it is pertinent to develop new therapeutic strategies for the treatment of gastric cancer.

In this study, we have established a novel anti-HER2 mAb through the immunization of mice with the purified recombinant protein for the HER2 extracellular domain. We subsequently investigated its use in a variety of applications, including flow cytometry, Western blot, and immunohistochemical analyses.

Materials and Methods

Cell lines

The following cell lines were all purchased from American Type Culture Collection (Manassas, VA): LN229, Chinese hamster ovary (CHO)-K1, and P3X63Ag8U.1 (P3U1). The cell lines MKN-7 and Kato III were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Miyagi, Japan). The amino acid sequences of tag system in this study were as follows: PA tag,[25] 12 amino acids (GVAMPGAEDDVV); RAP tag,[26] 12 amino acids (DMVNPGLEDRIE); and MAP tag,[27] 12 amino acids (GDGMVPPGIEDK). The cell lines LN229/HER2 and CHO/HER2 had been previously established by transfecting pCAG/PA-HER2-RAP-MAP into LN229 and CHO-K1 cells using the Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA) and Lipofectamine LTX (Thermo Fisher Scientific, Inc.), respectively.[28] LN229/$HER2_{ec}$ had been previously established by transfecting pCAG/PA-$HER2_{ec}$-RAP-MAP into LN229 cells using the Neon transfection system (Thermo Fisher Scientific, Inc.).[28] CHO-K1, CHO/HER2, P3U1, MKN-7, and Kato III were cultured in RPMI-1640 media (Nacalai Tesque, Inc., Kyoto, Japan), while LN229, LN229/HER2, and LN229/$HER2_{ec}$ were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Inc.). Media were supplemented with 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.), and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.) and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Hybridoma production

Female 4-week-old BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions, and the Animal Care and Use Committee of Tohoku University approved all animal experimentations described.

The ectodomain of HER2 with N-terminal PA tag and C-terminal RAP tag-MAP tag ($HER2_{ec}$) was purified from the culture supernatant of LN229/$HER2_{ec}$ using an anti-MAP tag mAb (clone PMab-1) as previously described.[28] Intraperitoneal injections were utilized to immunize BALB/c mice with 100 μg of $HER2_{ec}$ and Imject Alum (Thermo Fisher Scientific, Inc.). Additional immunizations were provided with a booster injection intraperitoneally administered 2 days before harvesting the spleen cells. Spleen cells were subsequently fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). RPMI-1640 medium, supplemented with 10% heat-inactivated 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.), and hypoxanthine/aminopterin/thymidine selection medium supplement (Thermo Fisher Scientific, Inc.), was used to culture the resulting hybridomas. Enzyme-linked immunosorbent assay (ELISA) was utilized to screen the culture supernatants with $HER2_{ec}$.

ELISA

A concentration of 1 μg/mL of $HER2_{ec}$ was immobilized on Nunc MaxiSorp 96-well immuno plates (Thermo Fisher Scientific, Inc.) for 30 minutes at 37°C. Plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST), and the wells were subsequently blocked with 1% bovine serum albumin (BSA; Nacalai Tesque, Inc.) containing PBST for 30 minutes at 37°C. The culture supernatant was then added to the plate along with a 1:2000 dilution of peroxidase-conjugated anti-mouse immunoglobulins (Agilent Technologies, Inc., Santa Clara, CA). We used 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.) for the enzymatic reactions. The optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Flow cytometric analyses

Cells were trypsinized with 0.25% trypsin in 1-mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.) and subsequently harvested. After washing with 0.1% BSA in PBS, cells were treated with 1 μg/mL of anti-HER2 mAb (H_2Mab-181) for 30 minutes at 4°C, with subsequent addition of Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using EC800 Cell Analyzers (Sony Corp., Tokyo, Japan).

Western blot analysis

Protein samples were obtained from cell lysates (10 μg) by boiling in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.) and electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and, subsequently, transferred onto polyvinylidene difluoride membranes (Merck KGaA, Darmstadt,

Germany). The membranes were blocked with 4% skim milk (Nacalai Tesque, Inc.) and then incubated with either 10 μg/mL of H_2Mab-181 or 1 μg/mL of anti-β-actin (clone AC-15; Sigma-Aldrich Corp., St. Louis, MO) and then with peroxidase-conjugated anti-mouse immunoglobulins (1:2000 dilution for H_2Mab-181, 1:1000 dilution for anti-β-actin; Agilent Technologies, Inc.). An ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) was used to develop the blots, and they were imaged with a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Determination of the binding affinity

Cells were suspended in 100 μL of serially diluted H_2Mab-181 (0.006–100 μg/mL). To this Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.) was added. Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp.). For the calculation of the dissociation constant (K_D), the binding isotherms were fitted to built-in one-site binding models in GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA).

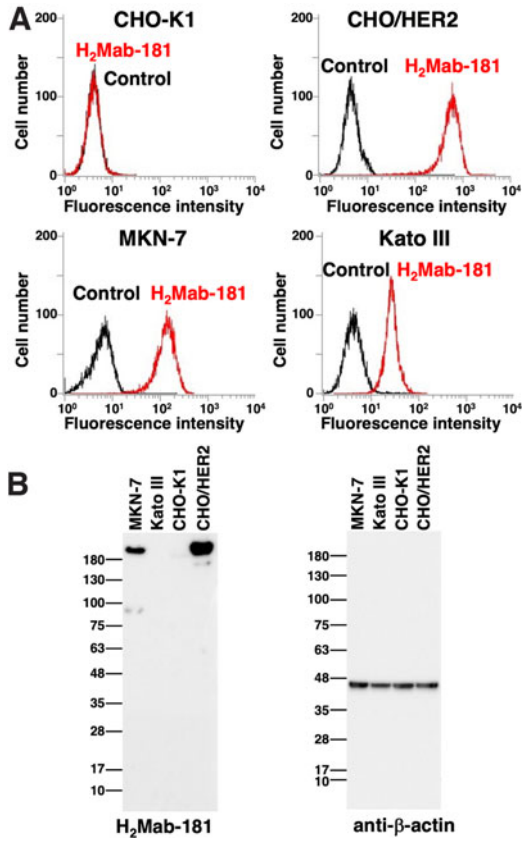

FIG. 1. Characterization of H_2Mab-181. **(A)** Flow cytometry with H_2Mab-181; CHO-K1, CHO/HER2, MKN-7, and Kato III were treated with 1 µg/mL of H_2Mab-181 followed by Alexa Fluor 488-conjugated anti-mouse IgG; the black line indicates the negative control. **(B)** Western blot using H_2Mab-181; cell lysates (10 µg) were electrophoresed, and proteins were transferred onto PVDF membranes. After blocking, membranes were incubated with 10 µg/mL of H_2Mab-181 or 1 µg/mL of anti-β-actin (AC-15) and then incubated with peroxidase-conjugated anti-mouse immunoglobulines. CHO, Chinese hamster ovary; HER2, human epidermal growth factor receptor 2; PVDF, polyvinylidene difluoride.

Immunohistochemical analyses

A microarray of the histologic sections (4 μm thickness) of stomach adenocarcinoma tissue (Catalog No.: BS01012e; US Biomax, Inc., Rockville, MD) was directly autoclaved in EnVision FLEX Target Retrieval Solution High pH (Agilent Technologies, Inc.) for 20 minutes. Sections were then blocked with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.) and then incubated with 5 μg/mL H_2Mab-181 for 1 hour at room temperature and then treated with an EnVision+ kit (Agilent Technologies, Inc.) for 30 minutes. The color was developed for 2 minutes using 3,3′-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.), and hematoxylin (FUJIFILM Wako Pure Chemical Corporation) was used to counterstain the sections. Hematoxylin and eosin (HE; FUJIFILM Wako Pure Chemical Corporation) staining was also performed. The intensity of staining was evaluated as -, 1+, 2+, 3+.

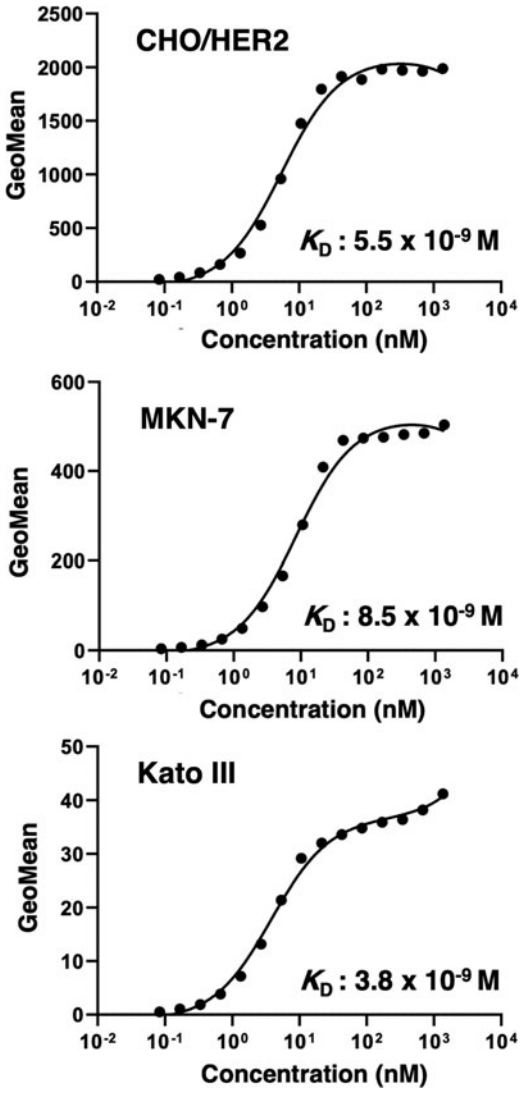

FIG. 2. Determination of the binding affinity by flow cytometry. CHO/HER2, MKN-7, and Kato III cells were suspended in 100 µL of serially diluted H_2Mab-181 (6 ng/mL to 100 µg/mL), and secondary anti-mouse IgG was subsequently added. Fluorescence data were collected using a cell analyzer.

Table 1. Immunohistochemical Analysis of Gastric Cancers Using H_2Mab-181

No.	*Sex*	*Age*	*Organ*	*Pathology diagnosis*	*TNM*	*Grade*	*Stage*	*Type*	*H_2Mab-181 intensity*
1	F	49	Stomach	Adenocarcinoma	T3N1M0	Moderately	IIB	Malignant	2+
2	M	68	Stomach	Adenocarcinoma	T3N0M0	Moderately	IIA	Malignant	3+
3	M	55	Stomach	Adenocarcinoma	T4N2M0	Moderately	IIIB	Malignant	—
4	M	75	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
5	F	60	Stomach	Adenocarcinoma	T2N1M0	Moderately	IIA	Malignant	—
6	F	48	Stomach	Adenocarcinoma	T3N2M0	Poorly	IIIA	Malignant	—
7	M	66	Stomach	Adenocarcinoma	T3N0M0	Moderately	IIA	Malignant	—
8	F	55	Stomach	Adenocarcinoma	T2N0M0	Well ~ moderately	IB	Malignant	—
9	F	47	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	2+
10	M	61	Stomach	Adenocarcinoma	T3N1M0	Moderately	IIB	Malignant	2+
11	M	70	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
12	M	60	Stomach	Adenocarcinoma	T2N1M0	Moderately	IIA	Malignant	—
13	M	54	Stomach	Adenocarcinoma	T2N1M0	Moderately	IIA	Malignant	—
14	M	72	Stomach	Adenocarcinoma	T2N1M0	Moderately	IIA	Malignant	—
15	M	71	Stomach	Adenocarcinoma	T2N1M0	Moderately	IIA	Malignant	2+
16	M	63	Stomach	Adenocarcinoma	T2N1M0	Moderately	IIA	Malignant	—
17	M	66	Stomach	Adenocarcinoma	T1N2M0	Moderately	IIA	Malignant	2+
18	M	53	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
19	F	68	Stomach	Adenocarcinoma	T2N1M0	Moderately	IIA	Malignant	—
20	F	64	Stomach	Adenocarcinoma	T2N1M0	Moderately	IIA	Malignant	—
21	F	50	Stomach	Adenocarcinoma	T3N0M0	Poorly	IIA	Malignant	—
22	M	72	Stomach	Adenocarcinoma	T2N0M0	Moderately ~ poorly	IB	Malignant	—
23	M	50	Stomach	Mucinous adenocarcinoma	T3N0M0	Poorly	IIA	Malignant	—
24	M	69	Stomach	Adenocarcinoma	T2N0M0	Poorly	IB	Malignant	—
25	M	59	Stomach	Adenocarcinoma	T2N0M0	Poorly	IB	Malignant	—
26	M	35	Stomach	Adenocarcinoma	T3N0M0	Poorly	IIA	Malignant	—
27	M	43	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
28	M	60	Stomach	Mucinous adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
29	F	70	Stomach	Adenocarcinoma	T2N0M0	Poorly	IB	Malignant	—
30	M	64	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	2+
31	M	45	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
32	F	35	Stomach	Adenocarcinoma	T3N2M0	Poorly	IIIA	Malignant	—
33	M	57	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
34	M	72	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
35	M	68	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
36	F	72	Stomach	Adenocarcinoma	T3N0M0	Poorly	IIA	Malignant	—
37	M	62	Stomach	Adenocarcinoma	T2N2M0	Poorly	IIB	Malignant	—
38	F	68	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	—
39	M	43	Stomach	Adenocarcinoma	T2N0M0	Poorly	IB	Malignant	—
40	M	55	Stomach	Adenocarcinoma	T3N2M0	Poorly	IIIA	Malignant	—
41	M	61	Stomach	Adenocarcinoma	T3N0M0	Poorly	IIA	Malignant	—
42	F	49	Stomach	Adenocarcinoma	T3N1M0	Moderately	IIB	Malignant	1+

(*continued*)

Table 1. (Continued)

No.	*Sex*	*Age*	*Organ*	*Pathology diagnosis*	*TNM*	*Grade*	*Stage*	*Type*	*H_2Mab-181 intensity*
43	M	49	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	3+
44	M	57	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
45	M	63	Stomach	Adenocarcinoma	T3N2M0	Poorly	IIIA	Malignant	—
46	M	60	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	—
47	F	46	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
48	M	50	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	—
49	M	63	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	—
50	F	37	Stomach	Adenocarcinoma	T2N2M0	Poorly	IIB	Malignant	—
51	M	45	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	—
52	M	34	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	—
53	M	54	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
54	M	64	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	—
55	M	40	Stomach	Adenocarcinoma	T3N2M0	Poorly	IIIA	Malignant	—
56	M	33	Stomach	Mucinous adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
57	M	70	Stomach	Adenocarcinoma	T2N0M0	Poorly	IB	Malignant	—
58	M	46	Stomach	Mucinous adenocarcinoma	T3N0M0	Poorly	IIA	Malignant	—
59	M	55	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
60	M	58	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	—
61	F	24	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
62	M	35	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
63	F	47	Stomach	Adenocarcinoma	T3N1M0	Poorly	IIB	Malignant	—
64	M	64	Stomach	Adenocarcinoma	T2N1M0	Poorly	IIA	Malignant	—
65	M	56	Stomach	Adenocarcinoma	T3N2M0	Moderately	IIIA	Malignant	3+
66	M	75	Stomach	Adenocarcinoma	T2N0M0	Poorly	IB	Malignant	—
67	M	48	Stomach	Adenocarcinoma	T2N0M0	Poorly	IB	Malignant	—
68	M	48	Stomach	Adenocarcinoma	T2N0M0	Poorly	IB	Malignant	—
69	F	74	Stomach	Adenocarcinoma	T2N0M0	Poorly	IB	Malignant	—
70	M	45	Stomach	Adenocarcinoma	T4N0M1	Poorly	IV	Malignant	—

Results

Establishment of an anti-HER2 mAb

Mice were immunized with the recombinant extracellular domain of HER2 ($HER2_{ec}$) and subsequently analyzed for anti-HER2 mAbs for binding to $HER2_{ec}$ using an ELISA. We next performed a flow cytometric analysis as a second evaluation to access the reaction in LN229 and LN229/HER2 cells. A stronger reaction against LN229/HER2 was necessary due to LN229 cells expressing endogenous HER2.[28] We finally established one clone H_2Mab-181 (IgG_1, kappa), which strongly reacted with LN229/HER2 and weakly with LN229 cells (data not shown).

Flow cytometric analyses

The flow cytometry data showed that H_2Mab-181 recognized CHO/HER2 cells but not CHO-K1 cells (Fig. 1A), which indicate that H_2Mab-181 is specific to HER2. H_2Mab-181 also recognized endogenous HER2 in MKN-7 and Kato III cells (Fig. 1A). The reaction of H_2Mab-181 in MKN-7 cells was higher than that for Kato III cells. These results show that H_2Mab-181 is suitable for the detection of gastric cancer cells.

Western blot analyses

H_2Mab-181 detected HER2 as a 180–200 kDa band in CHO/HER2 cells and did not detect any other bands within the CHO-K1 cells by Western blot analyses (Fig. 1B), which again suggests that H_2Mab-181 can specifically detect HER2 in Western blot analyses. H_2Mab-181 also detected endogenous HER2 in MKN-7 cells (Fig. 1B). In contrast, H_2Mab-181 did not detect HER2 in Kato III cells, which may be due to low HER2 expression level in these cells for the detection by H_2Mab-181 using Western blot analyses (Fig. 1B).

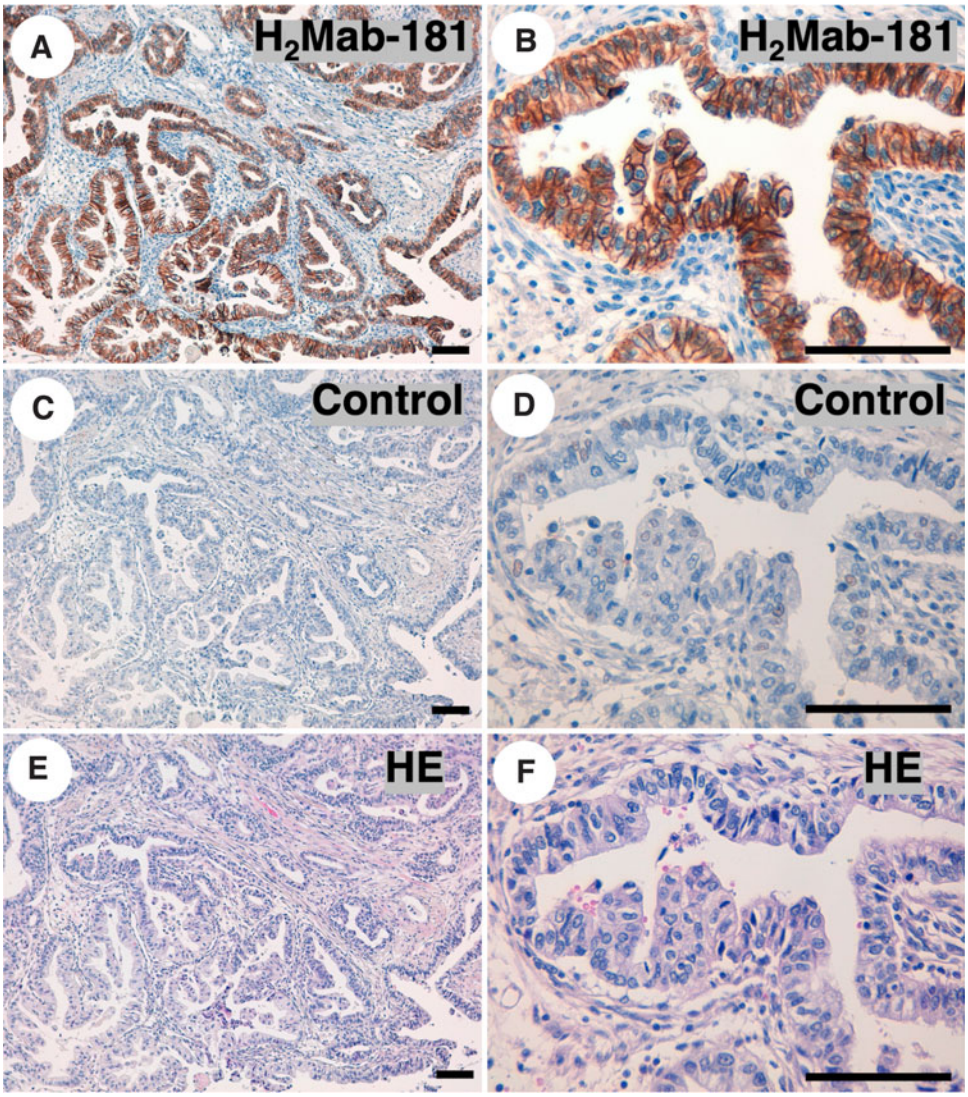

FIG. 3. Immunohistochemical analysis using H_2Mab-181. Histological sections of stomach adenocarcinoma tissue microarrays were directly autoclaved in EnVision FLEX Target Retrieval Solution High pH. After blocking, sections were incubated with 5 μg/mL of H_2Mab-181 **(A, B)** or blocking buffer control **(C, D)** and then treated with an EnVision+ kit. The color was developed using DAB. Sections were counterstained with hematoxylin. **(E, F)** HE staining was also performed. Scale bar = 100 μm. DAB, 3,3′-diaminobenzidine tetrahydrochloride; HE, hematoxylin and eosin.

Determination of the binding affinity

Kinetic analysis of the interactions between H_2Mab-181 and CHO/HER2, MKN-7, and Kato III cells was performed using flow cytometry. As shown in Figure 2, the dissociation constants (K_D) for the interaction of H_2Mab-181 with CHO/HER2, MKN-7, and Kato III cells were 5.5×10^{-9}, 8.5×10^{-9}, and 3.8×10^{-9} M, respectively, suggesting that H_2Mab-181 has a high affinity to HER2.

Immunohistochemical analyses

Finally, immunohistochemical analysis was performed using formalin-fixed paraffin-embedded tissue sections of stomach adenocarcinomas. Typical staining patterns (Patient No. 1 in Table 1) are shown in Figure 3A and B. The plasma membrane was strongly stained by H_2Mab-181 in the cancer cells. In 10 out of 70 cases (14.3%) of the stomach adenocarcinoma tissue array (Table 1), H_2Mab-181 staining was observed. No staining was observed in those sections stained with the buffer negative control (Fig. 3C, D). HE staining is shown in Figure 3E and F.

Discussion

The domain-I and domain-III of the extracellular region are ligand-binding sites for EGFR, HER3, and HER4. In contrast, no ligand can bind to HER2, and domain-I and -III of HER2 connect each other.[3,29] Heterodimers are formed between HER2 and other ligand-bound HER family members using largely domain-II-mediated dimerization interface, whereas heterodimerization with ligand-free HER family members mainly occurs through the domain IV.[30,31] Pertuzumab binds near the center of domain II of HER2 and blocks ligand-induced receptor dimerization and the subsequent signaling cascade.[32] Trastuzumab binds to the juxtamembrane region of domain IV, resulting in the inhibition of ligand-free receptor dimerization, stimulation of HER2 endocytosis, and removal of HER2 from the cell surface.[29] Pertuzumab and trastuzumab also possess antibody-dependent cellular cytotoxicity either alone or in combination with each other.[33]

Some studies have also previously reported the establishment of mAbs, which target domain III of HER2. Fu *et al.* developed a novel anti-HER2 mAb (hHERmAb-F0178C1) using the phage display method. hHERmAb-F0178C1 had efficacy in blocking HER2/HER3 heterodimerization and signaling, and its combinatory use with pertuzumab had a synergistic effect.[34] Meng *et al.* also established another novel anti-HER2 mAb (3E10) using hybridoma technology. In that study, 3E10 inhibited HER2 heterodimerization with EGFR and HER3 through a different mechanism to that of trastuzumab and pertuzumab. Synergistic inhibition of HER2 dimerization and signaling was observed with 3E10 in combination with either trastuzumab or pertuzumab. Moreover, triple-drug treatment of trastuzumab, pertuzumab, and 3E10 had even greater antitumor activity in HER2-overexpressing breast cancer models.[35] Together, these studies provide evidence that targeting the domain III of the HER2 extracellular region may be a new target for cancer treatment.

In this study, we have successfully developed a novel anti-HER2 mAb, H_2Mab-181, suitable for a range of applications, including flow cytometry, Western blot, and immunohistochemical analyses. Previously, we also established other anti-HER2 mAbs, such as H_2Mab-19,[36] H_2Mab-41,[37] H_2Mab-77,[28] H_2Mab-119,[38] and H_2Mab-139,[39] which are suitable for the same applications. However, the epitope of those anti-HER2 mAbs has not been clarified. For future studies, the epitope of H_2Mab-181 will be determined, and H_2Mab-181 will be investigated for antitumor activities within gastric cancers.

Author Disclosure Statement

No competing financial interests exist.

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