

## Development of an Anti-HER2 Monoclonal Antibody H2Mab-139 Against Colon Cancer

Mika K. Kaneko,<sup>1</sup> Shinji Yamada,<sup>1</sup> Shunsuke Itai,<sup>1</sup> and Yukinari Kato<sup>1,2</sup>

Human epidermal growth factor receptor 2 (HER2) expression has been reported in several cancers, such as breast, gastric, lung, pancreatic, and colorectal cancers. HER2 is overexpressed in those cancers and is associated with poor clinical outcomes. Trastuzumab, a humanized anti-HER2 antibody, provides significant survival benefits for patients with HER2-overexpressing breast cancers and gastric cancers. In this study, we developed a novel anti-HER2 monoclonal antibody (mAb), H<sub>2</sub>Mab-139 (IgG<sub>1</sub>, kappa) and investigated it against colon cancers using flow cytometry, western blot, and immunohistochemical analyses. Flow cytometry analysis revealed that H<sub>2</sub>Mab-139 reacted with colon cancer cell lines, such as Caco-2, HCT-116, HCT-15, HT-29, LS 174T, COLO 201, COLO 205, HCT-8, SW1116, and DLD-1. Although H<sub>2</sub>Mab-139 strongly reacted with LN229/HER2 cells on the western blot, we did not observe a specific signal for HER2 in colon cancer cell lines. Immunohistochemical analyses revealed sensitive and specific reactions of H<sub>2</sub>Mab-139 against colon cancers, indicating that H<sub>2</sub>Mab-139 is useful in detecting HER2 overexpression in colon cancers using flow cytometry and immunohistochemical analyses.

**Keywords:** HER2, monoclonal antibody, immunohistochemistry, colon cancer

### Introduction

HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2 (HER2) has been reported to be overexpressed in >20% of patients with breast cancer and is associated with poor clinical outcomes.<sup>(1,2)</sup> Trastuzumab and pertuzumab, humanized anti-HER2 monoclonal antibodies (mAbs), and trastuzumab emtansine, an antibody-drug conjugate, have been approved for the treatment of HER2-positive breast cancer.<sup>(3-5)</sup> Trastuzumab treatment has resulted in significant survival benefits for patients with metastatic HER2-positive breast cancer.<sup>(6)</sup> Furthermore, the combination of pertuzumab and trastuzumab with chemotherapy has led to significant improvements in overall survival compared with trastuzumab alone plus chemotherapy.<sup>(7)</sup> In addition, HER2 overexpression has been reported in gastric cancers,<sup>(8)</sup> lung cancers,<sup>(9)</sup> pancreatic cancers,<sup>(10)</sup> and colorectal cancers.<sup>(11)</sup> In this study, we developed a novel anti-HER2 mAb and characterized it against colon cancers using flow cytometry, western blot, and immunohistochemical analyses.

### Materials and Methods

#### Cell lines

LN229, Caco-2, HCT-116, HT-29, LS 174T, COLO 201, HCT-8, SW1116, and P3U1 cell lines were obtained from the

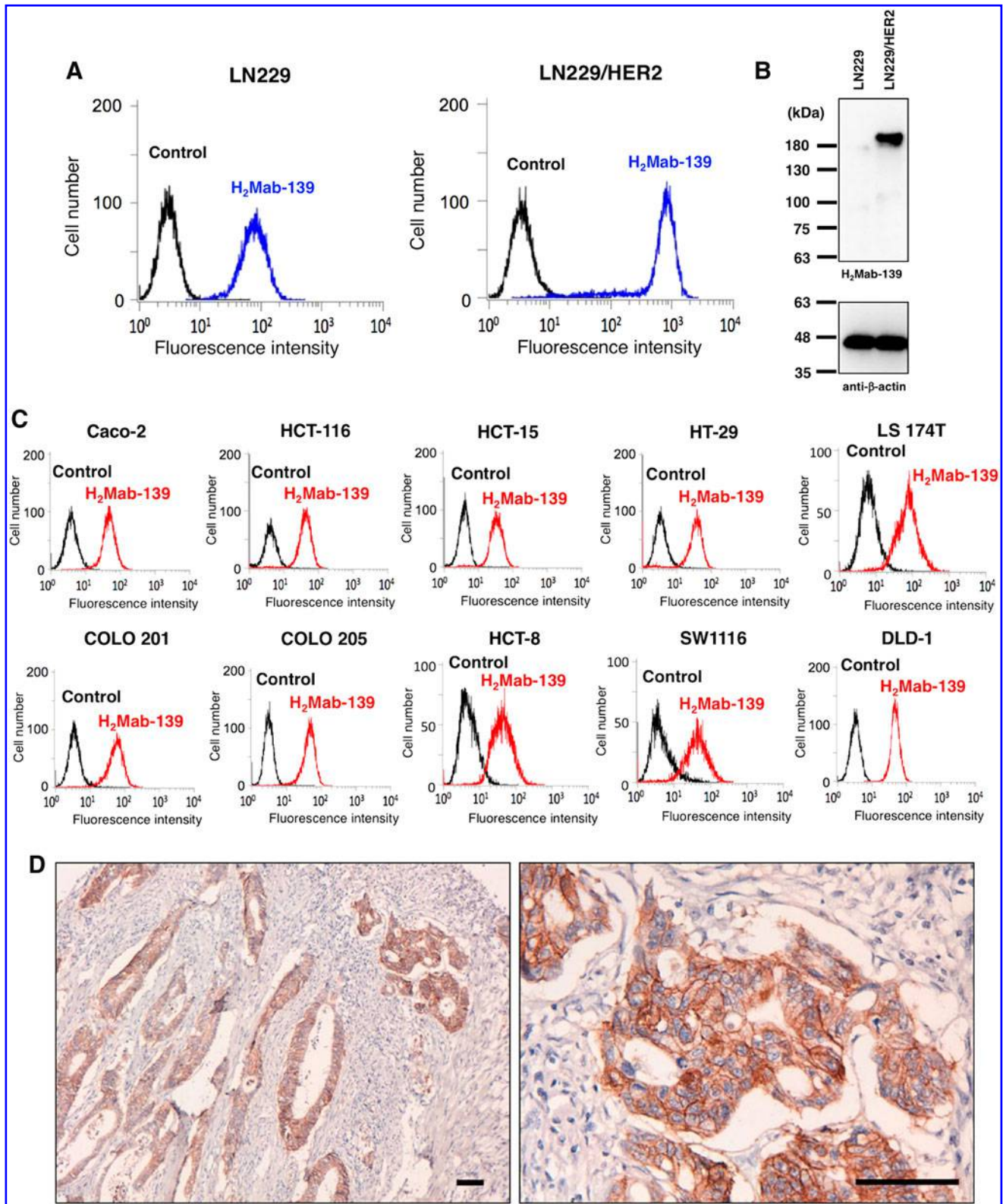
American Type Culture Collection (ATCC; Manassas, VA). COLO 205, DLD-1, and HCT-15 cell lines were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Miyagi, Japan). LN229/HER2 was produced previously.<sup>(12)</sup> COLO 205, DLD-1, HCT-15, COLO 201, SW1116, and P3U1 were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), and LN229, Caco-2, HCT-116, HT-29, LS 174T, and HCT-8 were cultured in DMEM (Dulbecco's modified Eagle's medium) medium (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA), 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 25 µg/mL of amphotericin B (Nacalai Tesque, Inc.). All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air.

#### Hybridoma production

Female 4-week-old BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions. The Animal Care and Use Committee of Tohoku University approved all the animal experiments described in this study. Anti-HER2 hybridomas were produced, as described previously.<sup>(13)</sup> Briefly, BALB/c mice were immunized using intraperitoneal (i.p.) injections of 100 µg of recombinant HER2-extracellular domain

<sup>1</sup>Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.

<sup>2</sup>New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.



**FIG. 1.** Characterization of H<sub>2</sub>Mab-139. (A) Flow cytometry with H<sub>2</sub>Mab-139; cells were treated with 10 μg/mL of H<sub>2</sub>Mab-139, followed by Alexa Fluor 488-conjugated anti-mouse IgG; black line, negative control. (B) Western blot using H<sub>2</sub>Mab-139; cell lysates were electrophoresed, and proteins were transferred onto PVDF membranes. After blocking, the membranes were incubated with 10 μg/mL of H<sub>2</sub>Mab-139 and 1 μg/mL of anti-β-actin (AC-15) and subsequently incubated with peroxidase-conjugated anti-mouse IgG. (C) Flow cytometry with H<sub>2</sub>Mab-139; colon cancer cell lines were treated with 10 μg/mL of H<sub>2</sub>Mab-139, followed by Alexa Fluor 488-conjugated anti-mouse IgG; black line, negative control. (D) Sections of colon cancer were incubated with 10 μg/mL of H<sub>2</sub>Mab-139 for 1 hour at room temperature, followed by treatment with Envision+ kit for 30 minutes. Color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB) for 2 minutes; subsequently, sections were counterstained with hematoxylin. Scale bar = 100 μm. HER2, human epidermal growth factor receptor 2; H<sub>2</sub>Mab-139, anti-HER2 monoclonal antibody; PVDF, polyvinylidene difluoride.

together with Imject Alum (Thermo Fisher Scientific, Inc.). After several additional immunizations, a booster injection was intraperitoneally administered 2 days before harvesting spleen cells. Spleen cells were then fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The resulting hybridomas were grown in RPMI medium supplemented with hypoxanthine, aminopterin, and thymidine selection medium supplement (Thermo Fisher Scientific, Inc.). Culture supernatants were screened using enzyme-linked immunosorbent assay with recombinant HER2-extracellular domain. MAbS were purified from the supernatants of hybridomas, cultured in Hybridoma-SFM medium (Thermo Fisher Scientific, Inc.) using Protein G Sepharose 4 Fast Flow (GE Healthcare UK, Ltd.).

#### Flow cytometry

Cells were harvested by brief exposure to 0.25% trypsin/1-mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin/phosphate-buffered saline, the cells were treated with 10  $\mu$ g/mL of anti-HER2 (clone H<sub>2</sub>Mab-139) for 30 minutes at 4°C and subsequently with 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.).

#### Western blot analysis

Cell lysates (10  $\mu$ g) were boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.) and proteins were then electrophoresed on 5%–20% polyacrylamide gels (Wako Pure Chemical Industries, Ltd., Osaka, Japan) before being transferred onto polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), membranes were incubated with primary antibodies, such as anti-HER2 (10  $\mu$ g/mL; clone H<sub>2</sub>Mab-139) and anti- $\beta$ -actin (1  $\mu$ g/mL; clone AC-15; Sigma-Aldrich, Corp., St. Louis, MO) and then with peroxidase-conjugated anti-mouse IgG (Agilent Technologies, Inc., Santa Clara, CA; 1:1000 diluted). Finally, membranes were developed using ImmunoStar LD (Wako Pure Chemical Industries, Ltd.) with a Sayaca-Imager (DRC, Co., Ltd., Tokyo, Japan).

#### Immunohistochemical analyses

Colon cancer tissue arrays were purchased from US Biomax, Inc. (Rockville, MD). Histologic sections of 4- $\mu$ m thickness were deparaffinized in xylene and subsequently rehydrated and autoclaved in EnVision FLEX Target Retrieval Solution, High pH (Agilent Technologies, Inc.), for 20 minutes. Sections were then incubated with 10  $\mu$ g/mL of H<sub>2</sub>Mab-139 for 1 hour at room temperature and treated using an Envision+ kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 2 minutes; subsequently, sections were counterstained with hematoxylin (Wako Pure Chemical Industries, Ltd.).

#### Results and Discussion

We immunized mice with the purified recombinant extracellular domain of HER2. We performed flow cytometry to

check reactions with LN229 and LN229/HER2 cells. A stronger reaction against LN229/HER2 was needed compared with LN229 because LN229 cells endogenously express HER2. We obtained one clone: H<sub>2</sub>Mab-139 (IgG<sub>1</sub>, kappa). Flow cytometry analysis revealed that H<sub>2</sub>Mab-139 reacted with LN229/HER2 cells more strongly than with endogenous HER2-expressing LN229 glioblastoma cells (Fig. 1A). H<sub>2</sub>Mab-139 also reacted with Chinese hamster ovary (CHO)/HER2 cells but did not react with CHO-K1 cells (data not shown), indicating that H<sub>2</sub>Mab-139 is specific for HER2. Western blot analysis of H<sub>2</sub>Mab-139 against LN229 and LN229/HER2 cells revealed that the molecular weight of H<sub>2</sub>Mab-139 was ~200 kDa in LN229/HER2 cells (Fig. 1B).

H<sub>2</sub>Mab-139 recognized endogenous HER2 in colon cancer cell lines, such as Caco-2, HCT-116, HCT-15, HT-29, LS 174T, COLO 201, COLO 205, HCT-8, SW1116, and DLD-1 (Fig. 1C). However, H<sub>2</sub>Mab-139 did not detect HER2 in colon cancer cell lines in western blots (data not shown), indicating that H<sub>2</sub>Mab-139 is not suitable for western blot analysis of colon cancer cell lines.

We further investigated the immunohistochemical utility of H<sub>2</sub>Mab-139 in human colon cancers. Figure 1D shows that H<sub>2</sub>Mab-139 stained the cell membranes of colon cancers that were previously diagnosed as HER2-positive. H<sub>2</sub>Mab-139 also stained the cell membranes of HER2-positive breast cancer cells (Supplementary Fig. S1), indicating that H<sub>2</sub>Mab-139 is useful for the immunohistochemical analysis for HER2-positive cancers. In the future, we intend to determine the positive rate of many colon cancer tissues via immunohistochemical analysis using H<sub>2</sub>Mab-139.

#### Acknowledgments

This work was supported in part by the Basic Science and Platform Technology Program for Innovative Biological Medicine from Japan Agency for Medical Research and Development, AMED (Y.K.), by Project for utilizing glycans in the development of innovative drug discovery technologies from AMED (Y.K.), and by Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research [BINDS]) from AMED (Y.K.).

#### Author Disclosure Statement

Y.K. received research funding from Ono Pharmaceutical Co., Ltd. All other authors have nothing to disclose.

#### References

1. Howlader N, Altekruse SF, Li CI, Chen VW, Clarke CA, Ries LA, and Cronin KA: US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. *J Natl Cancer Inst* 2014;106:dju055.
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, and McGuire WL: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987;235:177–182.
3. Lambert JM, and Chari RV: Ado-trastuzumab Emtansine (T-DM1): An antibody-drug conjugate (ADC) for HER2-positive breast cancer. *J Med Chem* 2014;57:6949–6964.
4. Valabrega G, Montemurro F, and Aglietta M: Trastuzumab: Mechanism of action, resistance and future perspectives in

- HER2-overexpressing breast cancer. *Ann Oncol* 2007;18:977–984.
5. Amiri-Kordestani L, Wedam S, Zhang L, Tang S, Tilley A, Ibrahim A, Justice R, Pazdur R, and Cortazar P: First FDA approval of neoadjuvant therapy for breast cancer: Pertuzumab for the treatment of patients with HER2-positive breast cancer. *Clin Cancer Res* 2014;20:5359–5364.
  6. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, and Norton L: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;344:783–792.
  7. Swain SM, Kim SB, Cortes J, Ro J, Semiglazov V, Campone M, Ciruelos E, Ferrero JM, Schneeweiss A, Knott A, Clark E, Ross G, Benyunes MC, and Baselga J: Pertuzumab, trastuzumab, and docetaxel for HER2-positive metastatic breast cancer (CLEOPATRA study): Overall survival results from a randomised, double-blind, placebo-controlled, phase 3 study. *Lancet Oncol* 2013;14:461–471.
  8. Xie S, Zhang H, Wang X, Ge Q, and Hu J: The relative efficacy and safety of targeted agents used in combination with chemotherapy in treating patients with untreated advanced gastric cancer: A network meta-analysis. *Oncotarget* 2017;8:26959–26968.
  9. Kim EK, Kim KA, Lee CY, and Shim HS: The frequency and clinical impact of HER2 alterations in lung adenocarcinoma. *PLoS One* 2017;12:e0171280.
  10. Harder J, Ihorst G, Heinemann V, Hofheinz R, Moehler M, Buechler P, Kloepfel G, Rocken C, Bitzer M, Boeck S, Endlicher E, Reinacher-Schick A, Schmoor C, and Geissler M: Multicentre phase II trial of trastuzumab and capecitabine in patients with HER2 overexpressing metastatic pancreatic cancer. *Br J Cancer* 2012;106:1033–1038.
  11. Seo AN, Kwak Y, Kim DW, Kang SB, Choe G, Kim WH, and Lee HS: HER2 status in colorectal cancer: Its clinical significance and the relationship between HER2 gene amplification and expression. *PLoS One* 2014;9:e98528.
  12. Fujii Y, Kaneko MK, and Kato Y: MAP tag: A novel tagging system for protein purification and detection. *Monoclon Antib Immunodiagn Immunother* 2016;35:293–299.
  13. Itai S, Fujii Y, Kaneko MK, Yamada S, Nakamura T, Yanaka M, Saidoh N, Chang YW, Handa S, Takahashi M, Suzuki H, Harada H, and Kato Y: H2Mab-77 is a sensitive and specific anti-HER2 monoclonal antibody against breast cancer. *Monoclon Antib Immunodiagn Immunother* 2017;36:143–148.

Address correspondence to:

*Yukinari Kato  
New Industry Creation Hatchery Center  
Tohoku University  
Department of Antibody Drug Development  
Tohoku University Graduate School of Medicine  
2-1 Seiryomachi  
Aoba-ku  
Sendai  
Miyagi 980-8575  
Japan*

*E-mail: yukinari-k@bea.hi-ho.ne.jp;  
yukinarikato@med.tohoku.ac.jp*

*Received: September 19, 2017  
Accepted: November 30, 2017*