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Short Communication

Establishment of H_2Mab-119, an Anti-Human Epidermal Growth Factor Receptor 2 Monoclonal Antibody, Against Pancreatic Cancer

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Human epidermal growth factor receptor 2 (HER2) is overexpressed in breast cancer and is associated with poor clinical outcomes. In addition, HER2 expression has been reported in other cancers, such as gastric, colorectal, lung, and pancreatic cancers. An anti-HER2 humanized antibody, trastuzumab, leads to significant survival benefits in patients with HER2-overexpressing breast cancers and gastric cancers. Herein, we established a novel anti-HER2 monoclonal antibody (mAb), H_2Mab-119 (IgG_1, kappa), and characterized its efficacy against pancreatic cancers using flow cytometry, Western blot, and immunohistochemical analyses. H_2Mab-119 reacted with pancreatic cancer cell lines, such as KLM-1, Capan-2, and MIA PaCa-2, but did not react with PANC-1 in flow cytometry analysis. Western blot analysis also revealed a moderate signal for KLM-1 and a weak signal for MIA PaCa-2, although H_2Mab-119 reacted strongly with LN229/HER2 cells. Finally, immunohistochemical analyses with H_2Mab-119 revealed sensitive and specific reactions against breast and colon cancers but did not react with pancreatic cancers, indicating that H_2Mab-119 is useful for detecting HER2 overexpression in pancreatic cancers using flow cytometry and Western blot analyses.

Keywords: HER2, monoclonal antibody, immunohistochemistry, pancreatic cancer

Introduction

Human epidermal growth factor receptor 2 (HER2) is involved in the progression of breast cancers.[1,2] HER2 overexpression is reported in >20% of patients with breast cancer and is associated with poor clinical outcomes. Humanized anti-HER2 monoclonal antibodies (mAbs), such as trastuzumab and pertuzumab; antibody-drug conjugate, including trastuzumab emtansine; and tyrosine kinase inhibitors, such as lapatinib, have been approved for the treatment of HER2-positive breast cancers.[3–6] Trastuzumab treatments have resulted in significant survival benefits in patients with metastatic HER2-positive breast cancers.[7] Furthermore, the combination of pertuzumab and trastuzumab with chemotherapy has led to significant improvements in overall survival compared with trastuzumab alone plus chemotherapy.[8] In addition, overexpression of HER2 has been reported in gastric cancers,[9] colorectal cancers,[10] lung cancers,[11] and pancreatic cancers.[12] In this study, we developed a novel anti-HER2 mAb and investigated its utility using flow cytometry, Western blot, and immunohistochemical analyses for pancreatic cancers.

Materials and Methods

Cell lines, tissues, and animals

LN229, Capan-2, and P3U1 cell lines were obtained from the American Type Culture Collection (Manassas, VA). KLM-1, PANC-1, and MIA PaCa-2 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 by transfecting pCAG/PA-HER2-RAP-MAP[13] into LN229 cells using the neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). A few days after transfection, PA tag-positive cells[14] were sorted using a cell sorter (SH800; Sony Corp., Tokyo, Japan). P3U1, KLM-1, and PANC-1 cell lines were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto,

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Japan); Capan-2 was cultured in McCoy's-5A medium (GE Healthcare UK Ltd., Buckinghamshire, England); whereas LN229, LN229/HER2, and MIA PaCa-2 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Nacalai Tesque, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/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_2 and 95% air. One breast cancer patient who underwent surgery at Sendai Medical Center was used for this study.[15] Colon cancer tissue arrays (40 cases) and pancreatic cancer tissue arrays (26 cases) were purchased from U.S. Biomax, Inc. (Rockville, MD). 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.

Hybridoma production

Anti-HER2 hybridomas were produced, as described previously.[15] In brief, BALB/c mice were immunized using intraperitoneal (i.p.) injections of 100 μg of recombinant HER2-extracellular domain 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 (ELISA) with recombinant HER2-extracellular domain. MAbs were purified from the supernatants of hybridomas, cultured in Hybridoma-SFM (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 μg/mL of anti-HER2 (clone H_2Mab-119) for 30 minutes at 4°C and subsequently with Alexa Fluor 488-conjugated antimouse 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 μg) 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 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 μg/mL; clone H_2Mab-119), anti-MAP tag (1 μg/mL; clone PMab-1),[13] and anti-β-actin (1 μg/mL, clone AC-15; Sigma-Aldrich Corp., St. Louis, MO) and then with peroxidase-conjugated antimouse IgG or antirat IgG (1:1000 diluted; Agilent Technologies, Inc., Santa Clara, CA). Finally, membranes were developed using ImmunoStar LD (Wako Pure Chemical Industries Ltd.) with a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Immunohistochemical analyses

Histological sections of 4 μm 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 μg/mL of H_2Mab-119 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 (Agilent Technologies, Inc.) for 2 minutes; subsequently, sections were counterstained with hematoxylin (Wako Pure Chemical Industries Ltd.).

Results and Discussion

In this study, we immunized mice with purified recombinant extracellular domain of HER2. Culture supernatants were then screened using ELISA. We used flow cytometry analyses to assess reactions with LN229 and LN229/HER2 cells. A stronger reaction against LN229/HER2 was necessary because LN229 cells express endogenous HER2. We obtained one clone H_2Mab-119 (IgG_1, kappa).

In flow cytometric analyses, H_2Mab-119 reacted with LN229/HER2 more strongly than with endogenous HER2-expressing LN229 glioblastoma cells (Fig. 1A). H_2Mab-119 also reacted with CHO/HER2 but did not react with the parental cell strain CHO-K1 (data not shown), indicating that H_2Mab-119 is specific for HER2. H_2Mab-119 recognized endogenous HER2 in pancreatic cancer cell lines, such as KLM-1, Capan-2, and MIA PaCa-2, but did not react with PANC-1 (Fig. 1B). In Western blots against LN229 and LN229/HER2 as well as pancreatic cancer cell lines, H_2Mab-119 detected a 180–200 kDa protein in LN229/HER2 (Fig. 1C). In contrast, H_2Mab-119 detected a moderate signal in KLM-1, a weak signal in MIA PaCa-2, and a faint signal in both PANC-1 and Capan-2, indicating that H_2Mab-119 is useful for Western blot analysis of pancreatic cancer cell lines.

Finally, we investigated the immunohistochemical utility of H_2Mab-119 in human breast cancers and pancreatic cancers. As shown in Figure 1D, H_2Mab-119 stained the cancer cell membranes of breast cancers and colon cancers that were diagnosed previously as HER2-positive breast cancer. In contrast, H_2Mab-119 did not stain HER2-positive pancreatic cancer tissues (data now shown), indicating that H_2Mab-119 is applicable only for flow cytometry and Western blot analyses against pancreatic cancers in this study, although it is useful for immunohistochemical analysis against breast cancers and colon cancers. In the future, we should stain many pancreatic cancer tissues to determine the positive rate in immunohistochemistry using H_2Mab-119.

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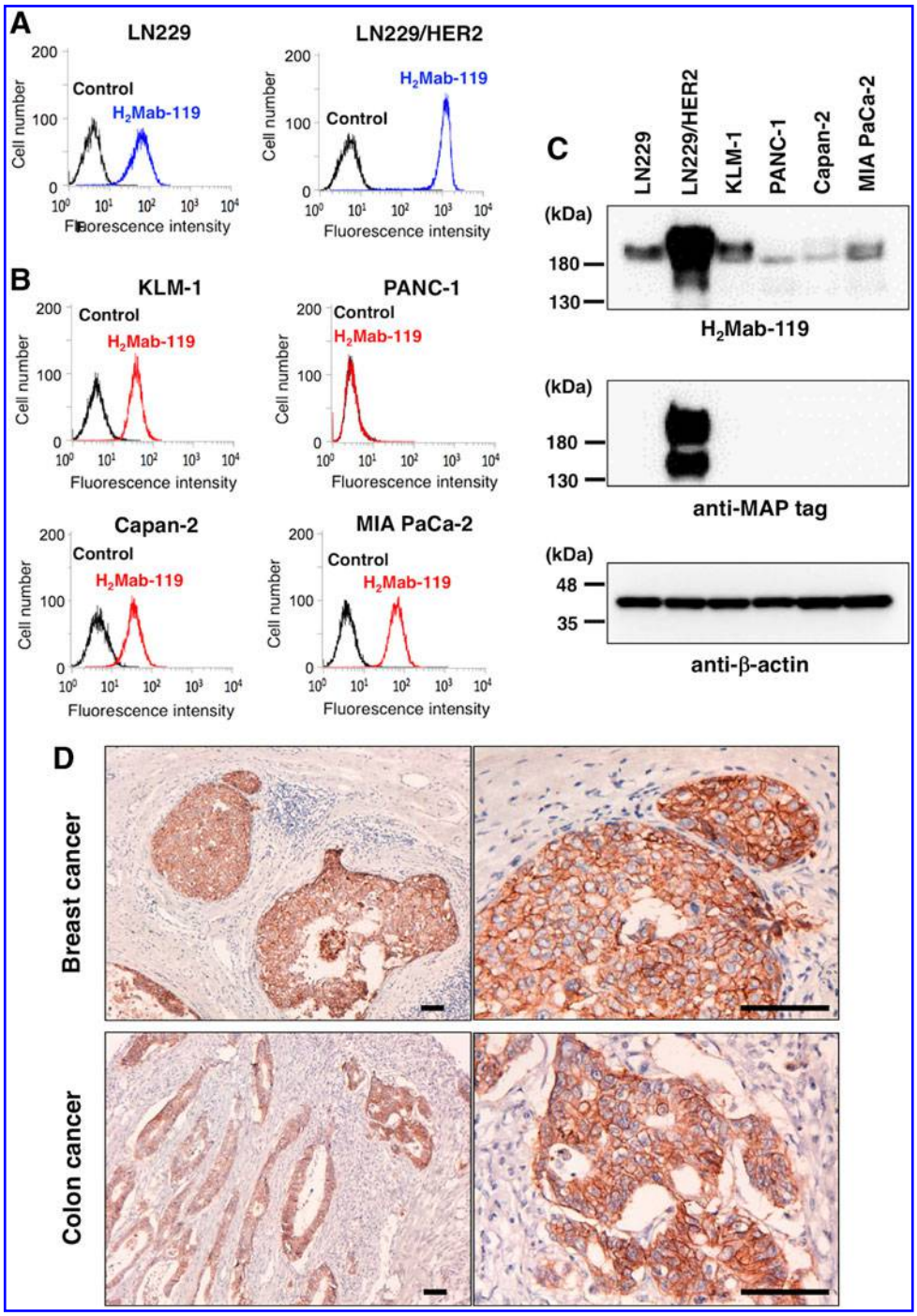

FIG. 1. Characterization of H_2Mab-119. **(A, B)** Flow cytometry with H_2Mab-119; cells were treated with 10 μg/mL of H_2Mab-119, followed by Alexa Fluor 488-conjugated anti-mouse IgG; black line, negative control. **(C)** Western blotting using H_2Mab-119; cell lysates were electrophoresed and proteins were transferred onto PVDF membranes. After blocking, membranes were incubated with 10 μg/mL of H_2Mab-119, 1 μg/mL of anti-MAP tag (PMab-1), or 1 μg/mL of anti-β-actin (AC-15) and subsequently incubated with peroxidase-conjugated antimouse IgG against H_2Mab-119 and AC-15 or antirat IgG against PMab-1. **(D)** Sections were incubated with 10 μg/mL of H_2Mab-119 for 1 hour at room temperature, followed by treatment with Envision+ kit for 30 minutes. Color was developed using 3,3-diaminobenzidine tetrahydrochloride for 2 minutes; subsequently, sections were counterstained with hematoxylin. Scale bar = 100 μm.

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Author Disclosure Statement

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