Received: 25 August 2023 | Revised: 14 October 2023 | Accepted: 22 October 2023

DOI: 10.1111/cas.16008

Cancer Science WILEY

ORIGINAL ARTICLE

# Antitumor activities against breast cancers by an afucosylated anti-HER2 monoclonal antibody $H_2$Mab-77-m$G_{2a}$-f

Tomohiro Tanaka[1] | Hiroyuki Suzuki[1,2] | Tomokazu Ohishi[3,4] | Mika K. Kaneko[1,2] | Yukinari Kato[1,2]

[1]Department of Molecular Pharmacology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

[2]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

[3]Institute of Microbial Chemistry (BIKAKEN), Numazu, Microbial Chemistry Research Foundation, Shizuoka, Japan

[4]Institute of Microbial Chemistry (BIKAKEN), Laboratory of Oncology, Microbial Chemistry Research Foundation, Tokyo, Japan

**Correspondence**
Hiroyuki Suzuki and Yukinari Kato, Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan.
Email: hiroyuki.suzuki.b4@tohoku.ac.jp and yukinari.kato.e6@tohoku.ac.jp

**Funding information**
Japan Agency for Medical Research and Development, Grant/Award Number: 23bm1123027h0001, JP23am0401013, JP23ama121008 and JP23ck0106730

## Abstract

Breast cancer patients with high levels of human epidermal growth factor receptor 2 (HER2) expression have worse clinical outcomes. Anti-HER2 monoclonal antibody (mAb) is the most important therapeutic modality for HER2-positive breast cancer. We previously immunized mice with the ectodomain of HER2 to create the anti-HER2 mAb, $H_2$Mab-77 (mouse $IgG_1$, kappa). This was then altered to produce $H_2$Mab-77-m$G_{2a}$-f, an afucosylated mouse $IgG_{2a}$. In the present work, we examined the reactivity of $H_2$Mab-77-m$G_{2a}$-f and antitumor effects against breast cancers in vitro and in vivo. BT-474, an endogenously HER2-expressing breast cancer cell line, was identified by $H_2$Mab-77-m$G_{2a}$-f with a strong binding affinity (a dissociation constant [$K_D$]: $5.0 \times 10^{-9}$ M). $H_2$Mab-77-m$G_{2a}$-f could stain HER2 of breast cancer tissues in immunohistochemistry and detect HER2 protein in Western blot analysis. Furthermore, $H_2$Mab-77-m$G_{2a}$-f demonstrated strong antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) for BT-474 cells. MDA-MB-468, a HER2-negative breast cancer cell line, was unaffected by $H_2$Mab-77-m$G_{2a}$-f. Additionally, in the BT-474-bearing tumor xenograft model, $H_2$Mab-77-m$G_{2a}$-f substantially suppressed tumor development when compared with the control mouse $IgG_{2a}$ mAb. In contrast, the HER2-negative MDA-MB-468-bearing tumor xenograft model showed no response to $H_2$Mab-77-m$G_{2a}$-f. These findings point to the possibility of $H_2$Mab-77-m$G_{2a}$-f as a treatment regimen by showing that it has antitumor effects on HER2-positive breast tumors.

**KEYWORDS**
ADCC, antitumor activity, CDC, HER2, monoclonal antibody

**Abbreviations:** ADC, antibody–drug conjugate; ADCC, antibody-dependent cellular cytotoxicity; CAR-T, chimeric antigen receptor-T; CDC, complement-dependent cytotoxicity; Fcγ, Fc-gamma; FDA, US Food and Drug Administration; HER2, human epidermal growth factor receptor 2; IDH1, isocitrate dehydrogenase 1; mAb, monoclonal antibody; NK, natural killer; PI3K, phosphoinositide 3-kinase; T-DXd, trastuzumab deruxtecan.

## 1 | INTRODUCTION

The receptor tyrosine kinase human epidermal growth factor receptor 2 (HER2, also known as ERBB2) is a type I transmembrane glycoprotein that controls cell development and survival. HER2 can form homodimers and heterodimers with related family members including EGFR, HER3, and HER4. The complexes initiate intracellular signaling pathways like mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K)/Akt. The extracellular domain of HER2 is composed of four regions, domain I–IV. Unlike other family receptors, HER2 extracellular domain basically has an active structure; dimer formation with other molecules is possible even without a ligand.[1,2] This structural characteristic is assumed to be the root of HER2's lack of ligand basis.[3] It should be mentioned that HER2 and HER3 interactions create extremely potent mitogenic signals and have been implicated in cancer progression.[4–6]

Twenty percent of breast cancer patients have an overexpression of HER2, which is linked to a poor prognosis.[7–10] Breast tumor incidence and development are correlated with HER2 gene amplification and protein overexpression.[11] Cell cycle progression, survival, angiogenesis, migration, invasion, and tumorigenesis are all accelerated by aberrant activation of HER2.[12–15] In order to forecast the effectiveness of anti-HER2 therapy, immunohistochemistry and/or in situ hybridization are often used to evaluate the HER2 status of breast cancer.[16] The development of HER2-targeted medicines has advanced significantly, and HER2 has grown in popularity as a cancer therapeutic target.[17]

Trastuzumab emtansine (T-DM1) and trastuzumab deruxtecan (T-DXd) are antibody–drug conjugates (ADCs) in which the payload is attached to trastuzumab, a humanized anti-HER2 monoclonal antibody (mAb).[18–20] Trastuzumab has been used to treat HER2-positive metastatic breast cancer. T-DM1 was authorized for HER2-positive advanced breast cancer by the US Food and Drug Administration (FDA) and the European Medicines Agency in 2013, while T-DXd was approved in the United States in 2019 for HER2-positive metastatic or unresectable breast cancer.[21–23] Trastuzumab binds to HER2 domain IV, causing antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell phagocytosis.[24–26] The extracellular domain of HER2 has been seen in breast cancer patients' serum, but trastuzumab binding prevents this HER2 cleavage.[27,28] After connecting to the receptors, the ADCs are internalized by endocytosis, where they eventually release their payloads and cause cell damage. As a result, trastuzumab-based ADCs are potent weapons that combine the dual antitumor action of trastuzumab with a cytotoxic payload. T-DM1 has been shown to preserve trastuzumab effects such as blocking HER2 ectodomain cleavage, inhibiting PI3K/Akt signaling, and engaging immune cells through Fc-gamma (Fcγ) receptors, resulting in ADCC.[29] T-DXd is an ADC of the future that combines deruxtecan and trastuzumab. Due to the bystander impact of its potent payload on surrounding cells, T-DXd has been found to be effective not only on HER2-high tumor cells but also on HER2-low tumor cells.[22,30] T-DXd is being tested in clinical studies to see whether it can be used to treat HER2-positive gastric and non-small cell lung cancer.[31,32]

Pertuzumab, a therapeutic antibody authorized in 2012 for HER2-positive breast cancer, binds to the domain II of HER2, preventing receptor dimerization with partner receptors and related signal transduction.[33–35] Pertuzumab, although having ADCC efficacy equal to trastuzumab, does not decrease HER2 shedding.[27,35,36] A potential mechanism for resistance to anti-HER2 therapy includes alteration of the HER2 ectodomain, which results in a reduction of anti-HER2 antibody binding affinity.[37] Anti-HER2 treatments using distinct anti-HER2 mAb variants, such as binding epitopes, or in conjunction with chemotherapy might pave the way for the continued development of HER2-targeted cancer therapies.

We previously created an anti-HER2 mAb (clone $H_2$Mab-77; mouse $IgG_1$, kappa) by immunizing mice with the ectodomain of HER2.[38] $H_2$Mab-77 could apply to flow cytometry, Western blotting, and immunohistochemical analyses. To test its ADCC, complement-dependent cytotoxicity (CDC), and antitumor efficacy in xenograft models, we also altered $H_2$Mab-77 into a core-fucose-deleted and subclass-converted anti-HER2 mAb ($H_2$Mab-77-m$G_{2a}$-f). The activation of natural killer (NK) cells, which is enhanced by the antibody's Fc region binding to FcγRIIIa on NK cells, results in the destruction of target cells, including tumor cells, and facilitates ADCC.[39] A core-fucose deletion in the Fc *N*-glycan has been shown to improve the binding of Fc to FcγRIIIa on effector NK cells.[40,41] This technique is also applied to clinically approved afucosylated mAb targeting CCR4, mogamulizumab (Potelligeo).[42] In this work, we assessed the antitumor efficacy of $H_2$Mab-77-m$G_{2a}$-f against breast cancers.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines

Human breast cancer cell lines (BT-474 and MDA-MB-468), a human glioblastoma cell line (LN229), and CHO-K1 were obtained from the American Type Culture Collection. The HER2-overexpressed CHO-K1 (CHO/HER2) and LN229 (LN229/HER2) cell lines were described previously.[38,43,44] CHO-K1 and CHO/HER2 cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc.). LN229, LN229/HER2, BT-474, MDA-MB-468, HEK293T, and HER2-knockout HEK293T (BINDS-23) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc.). Both media were supplemented with with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 μg/mL streptomycin, 100 units/mL penicillin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). The cells were maintained in a humidified incubator at 37°C with 5% $CO_2$ and 95% air atmosphere.

### 2.2 | Animals

The Institutional Committee for Experiments of the Institute of Microbial Chemistry (Numazu, Japan) authorized animal studies for

$H_2$Mab-77-m$G_{2a}$-f's antitumor efficacy (approval numbers 2022-056, 2023-001, and 2023-018). Humane objectives for euthanasia were established as a loss of original body weight to a point >25% and/or a maximal tumor size >3000 $mm^3$.

## 2.3 | Antibodies

We cloned the $V_H$ cDNA of $H_2$Mab-77 and the $C_H$ of mouse $IgG_{2a}$ into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation) in order to change the subclass of $H_2$Mab-77 from mouse $IgG_1$ to mouse $IgG_{2a}$ ($H_2$Mab-77-m$G_{2a}$). Additionally, the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation) was used to clone the $V_L$ cDNA of $H_2$Mab-77 and the $C_L$ cDNA of the mouse kappa light chain. The vector for $H_2$Mab-77-m$G_{2a}$ was transfected into BINDS-09, FUT8-knockout ExpiCHO-S cells. Using Ab-Capcher (ProteNova Co., Ltd.), $H_2$Mab-77-m$G_{2a}$-f, an afucosylated variant of the original antibody, was isolated. For the investigation of ADCC, CDC, and in vivo antitumor effectiveness, 281-m$G_{2a}$-f (an afucosylated anti-hamster podoplanin [PDPN] mAb) was employed as an afucosylated reference mouse $IgG_{2a}$.[45–47] Since 281-m$G_{2a}$-f does not recognize human PDPN, it does not react with human breast cancer cells (BT-474 and MDA-MB-468).

## 2.4 | Flow cytometry

Cells were harvested using 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). Subsequently, they were washed with 0.1% bovine serum albumin (Nacalai Tesque, Inc.) in phosphate-buffered saline (PBS), followed by treatment with $H_2$Mab-77-m$G_{2a}$-f (1 or 10 μg/mL) for 30 min at 4°C. Then, cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000 dilution; Cell Signaling Technology, Inc.), and fluorescence was measured using an SA3800 Cell Analyzer (Sony Corp.).

## 2.5 | Determination of the binding affinity by enzyme-linked immunosorbent assay (ELISA) and flow cytometry

The serially diluted $H_2$Mab-77 and $H_2$Mab-77-m$G_{2a}$-f (0.0006–10 μg/mL) were added to HER2 ectodomain (HER2ec)-immobilized wells, followed by peroxidase-conjugated anti-mouse immunoglobulins (1:3000 diluted; Agilent Technologies Inc.). Enzymatic reactions were conducted using 1 Step Ultra TMB (Thermo Fisher Scientific Inc.) followed by the measurement of optical density at 655 nm, using an iMark microplate reader (Bio-Rad Laboratories, Inc.).

After being suspended in 100 μL of serially diluted $H_2$Mab-77-m$G_{2a}$-f (0.0006–10 μg/mL), the cells were incubated with 1:200 of Alexa Fluor 488-conjugated anti-mouse IgG. The SA3800 Cell Analyzer (Sony Corp.) flow cytometer was then used to gather fluorescence data.

The binding isotherms were fitted into the built-in, one-site binding model in GraphPad PRISM 6 (GraphPad Software, Inc.) to calculate the binding affinity.

## 2.6 | Western blotting

Western blotting was performed using $H_2$Mab-77-m$G_{2a}$-f (1 μg/mL) and an anti-isocitrate dehydrogenase 1 (IDH1) mAb, RcMab-1 (1 μg/mL), as described previously.[38]

## 2.7 | Immunohistochemical analysis

After being autoclaved in EnVision FLEX Target Retrieval Solution High pH (Agilent Technologies, Inc.) for 20 min, paraffin-embedded tissue sections from the breast cancer tissue array (Cat. No. T8235721-5, BioChain) were treated with 3% hydrogen peroxide for 15 min at room temperature. After blocking using SuperBlock T20 (Thermo Fisher Scientific, Inc.), the sections were incubated with $H_2$Mab-77-m$G_{2a}$-f (10 μg/mL) for 60 min and then with the EnVision+ Kit for mouse (Agilent Technologies, Inc.) for 30 min. The chromogenic reaction was performed as described previously.[38]

## 2.8 | Antibody-dependent cellular cytotoxicity reporter bioassay

The ADCC reporter bioassay was performed using an ADCC Reporter Bioassay kit from Promega, following the manufacturer's instructions. Target cells (12,500 cells per well) were inoculated into a 96-well white solid plate. $H_2$Mab-77-m$G_{2a}$-f, $H_2$Mab-77, and 281-m$G_{2a}$-f were serially diluted and added to the target cells. Jurkat cells stably expressing the human FcγRIIIa receptor, and a nuclear factor of activated T cells (NFAT) response element driving firefly luciferase, were used as effector cells. The engineered Jurkat cells (75,000 cells in 25 μL) were then added and cocultured with antibody-treated target cells at 37°C for 6 h. Luminescence using the Bio-Glo Luciferase Assay System (Promega) was measured with a GloMax luminometer (Promega).

## 2.9 | Antibody-dependent cellular cytotoxicity of $H_2$Mab-77-m$G_{2a}$-f

The ADCC activity of $H_2$Mab-77-m$G_{2a}$-f was measured as described previously.[48] Effector cells were obtained from the spleen of female BALB/c nude mice (Charles River Laboratories). In brief, the spleens were processed through a sterile cell strainer (352360, BD Falcon) to produce single-cell suspensions. A 10-s exposure to ice-cold, distilled water destroyed erythrocytes. Effector cells were washed with DMEM and resuspended in DMEM with 10% FBS. Calcein AM (10 μg/mL) was used to mark the target cells (BT-474 and MDA-MB-468). In

96-well plates, target cells ($2\times10^4$ cells) were seeded before effector cells (effector to target ratio, 50:1) and 100 μg/mL of either $H_2$Mab-77-$mG_{2a}$-f or 281-$mG_{2a}$-f was added. A microplate reader (Power Scan HT; BioTek Instruments, Inc.) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm was used to analyze the calcein release into the medium after a 4.5-h incubation at 37°C.

This is how cytolyticity (% lysis) was determined: % lysis is calculated as $(E-S)/(M-S)\times100$, where $E$ denotes the fluorescence in effector and target cell cultures, $S$ denotes the spontaneous fluorescence of only target cells, and $M$ denotes the maximum fluorescence after treatment with a lysis buffer (10 mM Tris–HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100).

### 2.10 | Complement-dependent cytotoxicity of $H_2$Mab-77-$mG_{2a}$-f

The following procedure was used to assess how well $H_2$Mab-77-$mG_{2a}$-f induced CDC. With 10 μg/mL Calcein AM, the target cells (BT-474 and MDA-MB-468) were marked. Target cells ($2\times10^4$ cells) were put in 96-well plates with 100 μg/mL of either $H_2$Mab-77-$mG_{2a}$-f or 281-$mG_{2a}$-f and rabbit complement (final dilution 1:10; Low-Tox-M Rabbit Complement; Cedarlane Laboratories). The amount of calcein released into the medium was measured during 4.5-h incubation at 37°C.

### 2.11 | Antitumor activities of $H_2$Mab-77-$mG_{2a}$-f in xenografts of breast cancer

BT-474 and MDA-MB-468 were mixed with DMEM and BD Biosciences' Matrigel Matrix Growth Factor Reduced. Subcutaneous injections were then given to the left flanks of BALB/c nude mice. On the seventh post-inoculation day, 100 μg of $H_2$Mab-77-$mG_{2a}$-f ($n=8$) or 281-$mG_{2a}$-f ($n=8$) in 100 μL PBS was administered intraperitoneally. Additional antibody injections were given on days 14 and 21. The tumor diameter was assessed on days 7, 10, 14, 16, 21, 24, and 28 after breast cancer cell implantation. Tumor volumes were calculated in the same manner as previously stated. The weight of the mice was also assessed on days 7, 10, 14, 16, 21, 24, and 28 following breast cancer cell inoculation. When the observations were finished on day 28, the mice were sacrificed, and tumor weights were assessed following tumor excision.

### 2.12 | Statistical analyses

All data are represented as mean ± standard error of the mean (SEM). Welch's $t$-test was conducted for ADCC activity, CDC activity, and tumor weight. ANOVA with Sidak's post hoc test was conducted for tumor volume and mouse weight. GraphPad Prism 8 (GraphPad Software, Inc.) was used for all calculations. $p<0.05$ was considered to indicate a statistically significant difference.

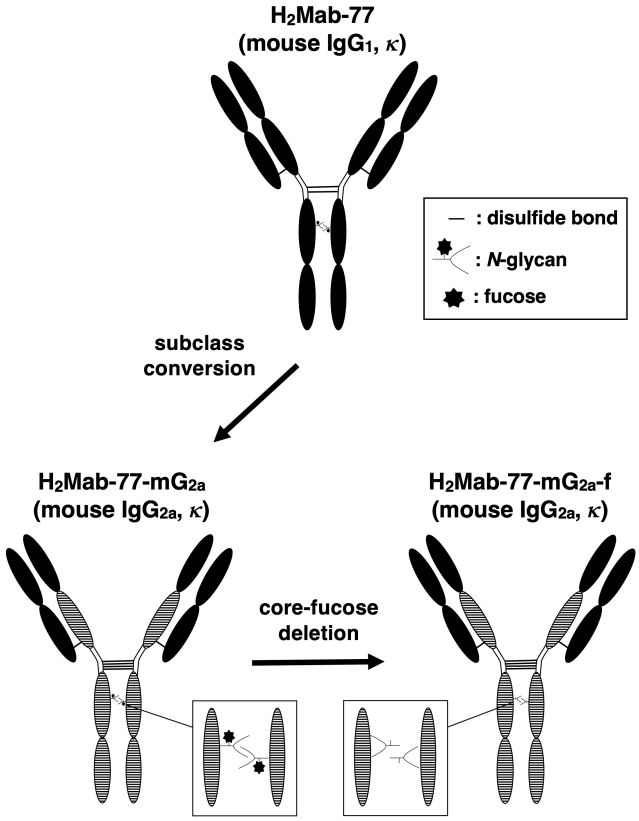


FIGURE 1 Generation of $H_2$Mab-77-$mG_{2a}$-f (mouse $IgG_{2a}$) and $H_2$Mab-77-$mG_{2a}$-f (core-fucose-deleted mouse $IgG_{2a}$) from the original anti-HER2 mAb, $H_2$Mab-77 (mouse $IgG_1$).

## 3 | RESULTS

### 3.1 | Development of a core-fucose-deficient anti-HER2 mAb, $H_2$Mab-77-$mG_{2a}$-f

We previously created an anti-HER2 mAb, $H_2$Mab-77 (mouse $IgG_1$, kappa), by immunizing mice with the HER2 ectodomain. Flow cytometry, Western blotting, and immunohistochemical tests revealed that $H_2$Mab-77 has a high binding affinity.[38]

In this study, we changed the subclass of antibody to improve its antitumor efficacy. We altered the subclass of $H_2$Mab-77 from mouse $IgG_1$ to mouse $IgG_{2a}$ by joining the $V_H$ chain of $H_2$Mab-77 with the $C_H$ chain of mouse $IgG_{2a}$ because mouse $IgG_1$ has no ADCC and CDC activity. In addition, utilizing BINDS-09 cells (ExpiCHO-S cells lacking FUT8 fucosyltransferase), an afucosylated mouse $IgG_{2a}$ form of $H_2$Mab-77 ($H_2$Mab-77-$mG_{2a}$-f) was generated (Figure 1).

### 3.2 | Analysis of $H_2$Mab-77-$mG_{2a}$-f reactivity against breast cancer cells by flow cytometry

In a previous study, the original $H_2$Mab-77 (mouse $IgG_1$, kappa) was found to be suitable for flow cytometry, Western blotting, and immunohistochemistry.[38] In this study, an afucosylated form of the anti-HER2 mAb, $H_2$Mab-77-$mG_{2a}$-f, was generated. Similar

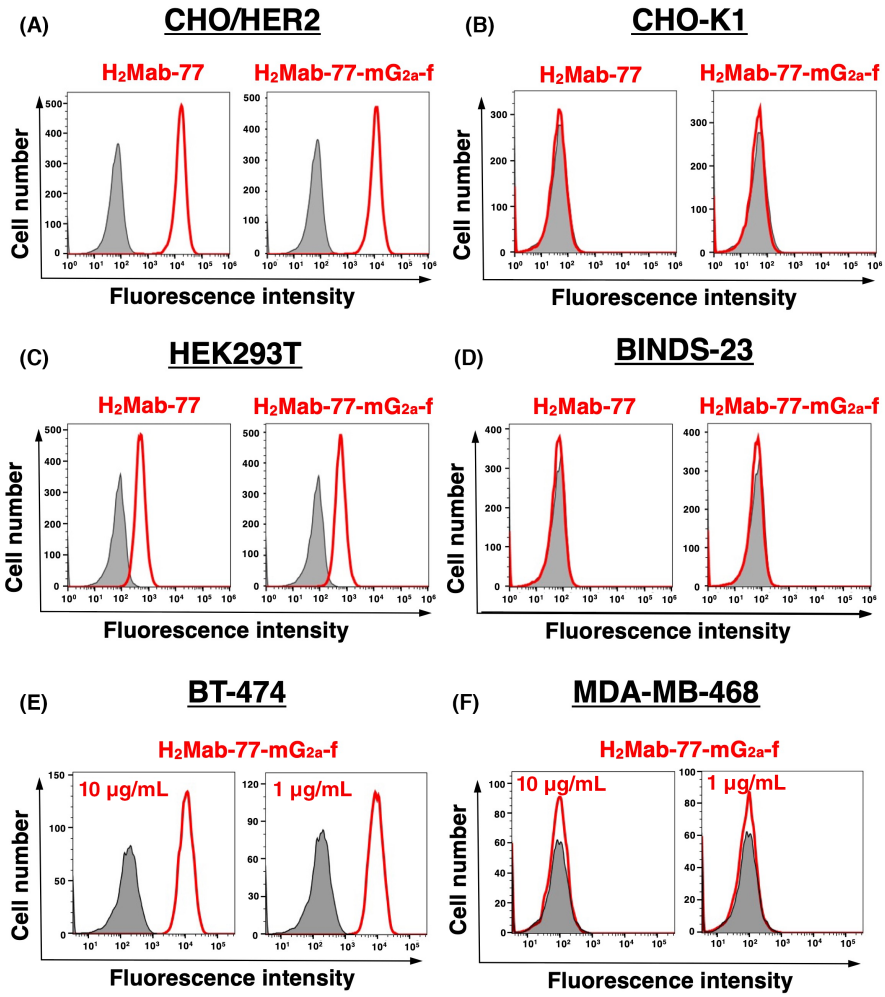


FIGURE 2 Flow cytometric analysis using $H_2$Mab-77-m$G_{2a}$-f. (A–D) CHO/HER2 (A), CHO-K1 (B), HEK293T (C), and HER2-knockout HEK293T (BINDS-23) (D) cells were treated with $H_2$Mab-77-m$G_{2a}$-f or $H_2$Mab-77 (red) or buffer control (black), followed by Alexa Fluor 488-conjugated anti-mouse IgG. (E, F) BT-474 (E) and MDA-MB-468 (F) cells were treated with $H_2$Mab-77-m$G_{2a}$-f followed by Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were analyzed using the SA3800 Cell Analyzer.

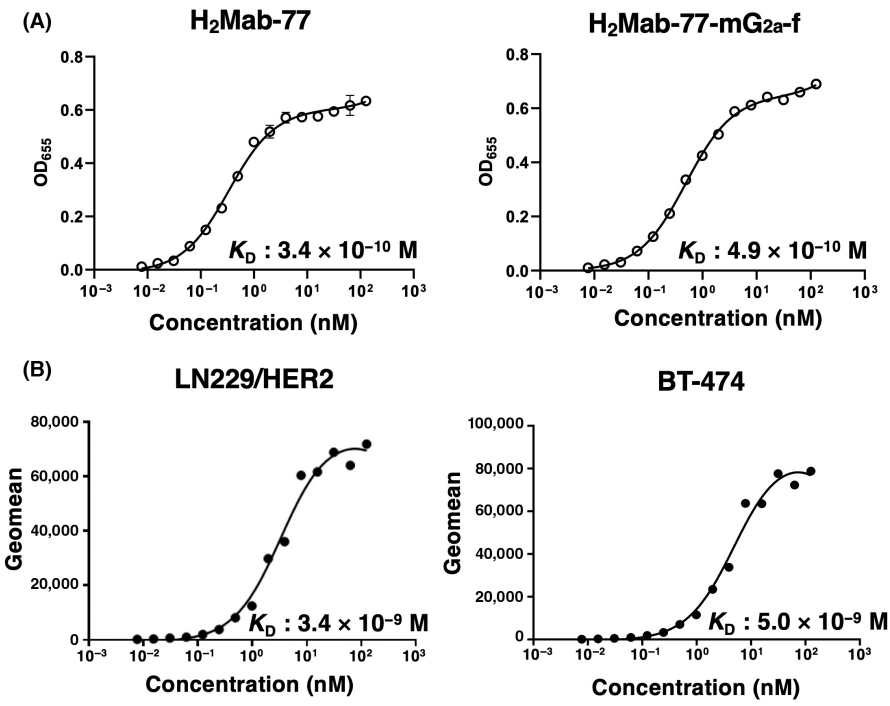


FIGURE 3 Determination of the binding affinity of $H_2$Mab-77-m$G_{2a}$-f by ELISA and flow cytometry. (A) HER2ec was immobilized on immunoplates and then incubated with the serially diluted $H_2$Mab-77 and $H_2$Mab-77-m$G_{2a}$-f (0.0006–10 μg/mL), followed by peroxidase-conjugated anti-mouse immunoglobulins. Enzymatic reactions were conducted, and the dissociation constant ($K_D$) was calculated using the optical density at 655 nm. (B) The binding affinity of $H_2$Mab-77-m$G_{2a}$-f was determined by flow cytometry in LN229/HER2 and BT-474 cells. Serially diluted $H_2$Mab-77-m$G_{2a}$-f was added to the cells, followed by Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer, and the $K_D$ was calculated.

to the original $H_2$Mab-77, $H_2$Mab-77-m$G_{2a}$-f successfully detected HER2 in HER2-overexpressed Chinese hamster ovary (CHO)-K1 (CHO/HER2) cells (Figure 2A), but not in CHO-K1 cells (Figure 2B). Furthermore, both $H_2$Mab-77 and $H_2$Mab-77-m$G_{2a}$-f reacted with HEK293T cells (Figure 2C), but not with HER2-knockout HEK293T (BINDS-23) cells (Figure 2D).

Next, we conducted flow cytometric analysis using $H_2$Mab-77-m$G_{2a}$-f on two breast cancer cell lines, BT-474 and MDA-MB-468. BT-474 represents the luminal B type of breast cancer with positive expression of progesterone receptor ($PR^+$), estrogen receptor ($ER^+$), and HER2. In contrast, MDA-MB-468 is a triple-negative breast cancer lacking expression of PR, ER, and HER2.[49] $H_2$Mab-77-m$G_{2a}$-f demonstrated recognition of endogenously expressed HER2 in BT-474 at 1 or 10 μg/mL, while it showed no response to MDA-MB-468 at either concentration (Figure 2E,F).

### 3.3 | Determination of the binding affinity of $H_2$Mab-77-m$G_{2a}$-f to HER2 by ELISA and flow cytometry

We next evaluated the binding affinity of $H_2$Mab-77 and $H_2$Mab-77-m$G_{2a}$-f to purified HER2ec by ELISA. As shown in Figure 3A, $H_2$Mab-77 and $H_2$Mab-77-m$G_{2a}$-f showed comparable dissociation constant ($K_D$) to HER2ec as $3.4\times10^{-10}$ and $4.9\times10^{-10}$ M, respectively. We next investigated the binding affinity of $H_2$Mab-77-m$G_{2a}$-f using flow cytometry. The $K_D$ of $H_2$Mab-77-m$G_{2a}$-f to LN229/HER2 and BT-474 cells were determined to be $3.4\times10^{-9}$ and $5.0\times10^{-9}$ M, respectively (Figure 3B). These findings indicate that $H_2$Mab-77-m$G_{2a}$-f can effectively recognize HER2 with a high binding affinity.

### 3.4 | Western blot and immunohistochemical analyses using $H_2$Mab-77-m$G_{2a}$-f

Similar to the original $H_2$Mab-77, $H_2$Mab-77-m$G_{2a}$-f detected the HER2 band with an estimated 180-kDa band in lysates generated from LN229, LN229/HER2, and BT-474 cells; no band was seen in MDA-MB-468 cells, which are HER2 negative (Figure 4A). For the internal control, an anti-IDH1 mAb (clone RcMab-1) was employed (Figure 4B). These findings revealed that $H_2$Mab-77-m$G_{2a}$-f may be employed in Western blotting to assess HER2 expression in cultured cells, including breast cancer cells.

The $H_2$Mab-77-m$G_{2a}$-f antibody was then utilized to target clinical specimens of a human breast cancer tissue array via immunohistochemistry analysis. Similar to the original $H_2$Mab-77, $H_2$Mab-77-m$G_{2a}$-f identified HER2 on the plasma membrane of breast cancer tissue (Figure 4C,D). Table S1 summarizes the immunohistochemical study of breast cancer. $H_2$Mab-77-m$G_{2a}$-f effectively stained HER2 in 14 of 63 cases (22%) of breast cancer, showing its use in the immunohistochemical investigation of formalin-fixed paraffin-embedded tumor sections for identifying HER2.

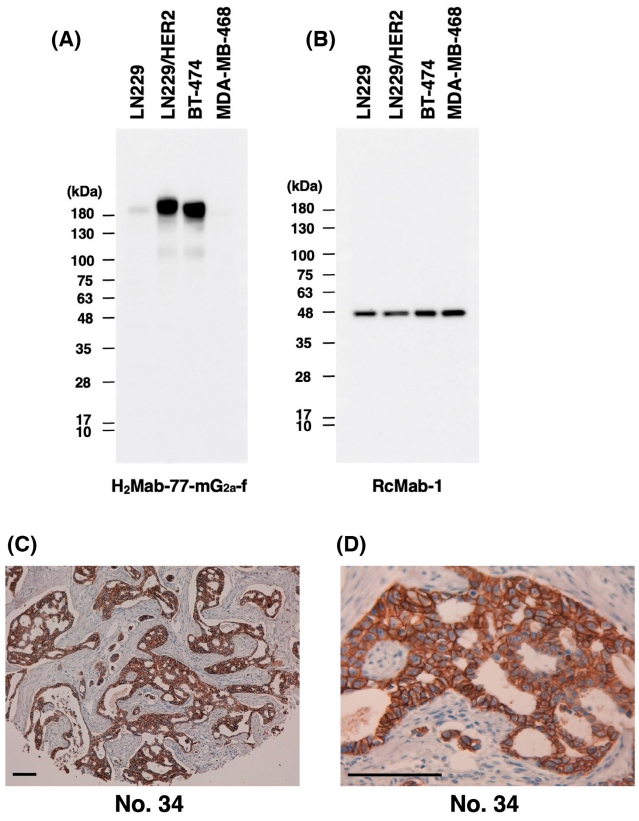


FIGURE 4 Detection of HER2 by Western blotting using $H_2$Mab-77-m$G_{2a}$-f. Cell lysates were electrophoresed and transferred onto PVDF membranes. After blocking, the PVDF membranes were incubated with $H_2$Mab-77-m$G_{2a}$-f (1 μg/mL) (A) or an anti-IDH1 monoclonal antibody (clone RcMab-1, 1 μg/mL) (B), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins or peroxidase-conjugated anti-rat immunoglobulins. (C, D) Detection of HER2 in breast cancer specimens using immunohistochemical analysis with $H_2$Mab-77-m$G_{2a}$-f. Tissue sections of human breast cancer were incubated with $H_2$Mab-77-m$G_{2a}$-f at a concentration of 10 μg/mL and then treated with the EnVision+ kit. Scale bar: 100 μm.

### 3.5 | Effector cell activation by $H_2$Mab-77-m$G_{2a}$-f against HER2-positive cells

The ADCC reporter bioassay is a bioluminescent reporter gene assay to quantify the biological activity of the antibody via FcγRIIIa-mediated pathway activation in an ADCC mechanism of action. To compare the ADCC pathway activation by $H_2$Mab-77-m$G_{2a}$-f and $H_2$Mab-77, we treated CHO/HER2 and BT-474 cells with serially diluted mAbs, and then incubated them with effector Jurkat cells, which express the human FcγRIIIa receptor and an NFAT response element driving firefly luciferase reporter. Furthermore, we also used afucosylated anti-hamster PDPN mAb

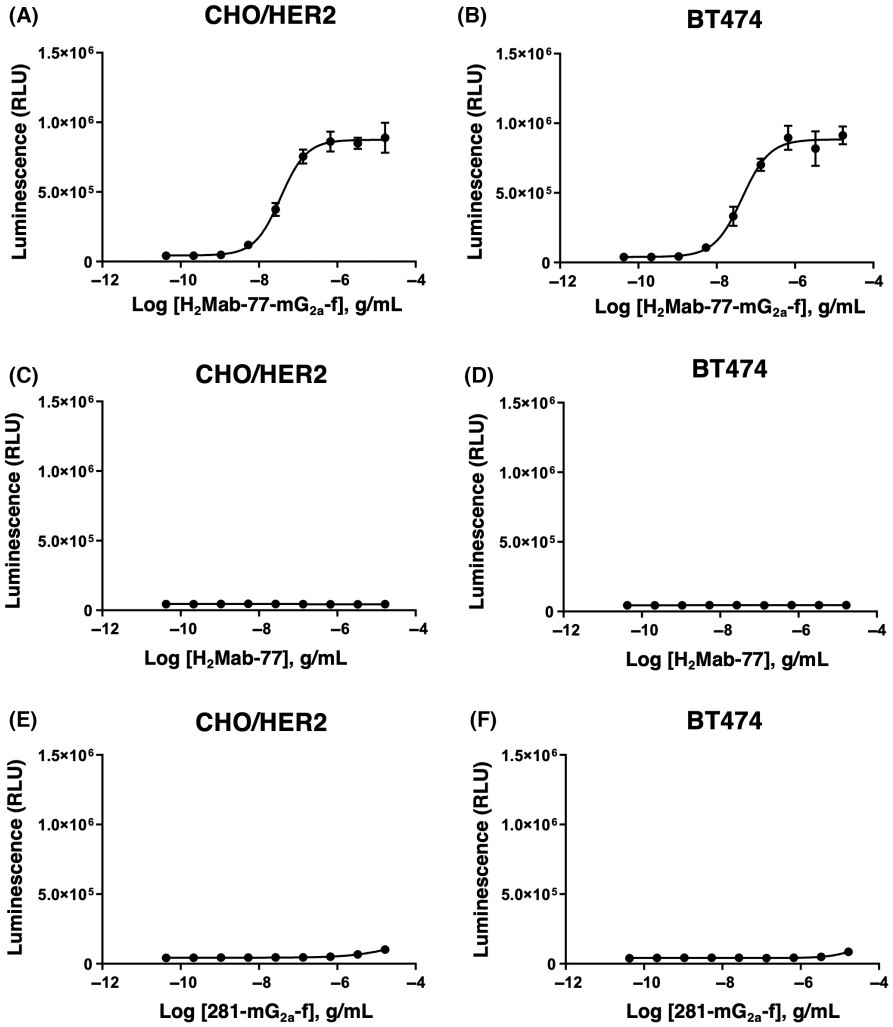


**FIGURE 5** Antibody-dependent cellular cytotoxicity (ADCC) reporter assay by $H_2$Mab-77-m$G_{2a}$-f, $H_2$Mab-77, and 281-m$G_{2a}$-f in the presence of CHO/HER2 and BT-474 cells. (A, B) Target cells (CHO/HER2 [A] and BT-474 [B]) were treated with serially diluted $H_2$Mab-77-m$G_{2a}$-f. (C, D) Target cells (CHO/HER2 [C] and BT-474 [D]) were treated with serially diluted $H_2$Mab-77. (E, F) Target cells (CHO/HER2 [E] and BT-474 [F]) were treated with serially diluted 281-m$G_{2a}$-f (mouse afucosylated $IgG_{2a}$ control). Then, Jurkat cells stably expressing the human FcγRIIIa receptor and an NFAT response element driving firefly luciferase reporter were added and cocultured. Luminescence was measured using the Bio-Glo Luciferase Assay System.

(mouse $IgG_{2a}$; 281-m$G_{2a}$-f) as a control. As shown in Figure 5A, $H_2$Mab-77-m$G_{2a}$-f dose-dependently activated the effector in the presence of CHO/HER2 cells ($EC_{50}$; 35.7 ng/mL; Figure 5A) and BT-474 cells ($EC_{50}$; 44.4 ng/mL; Figure 5B). We confirmed that original $H_2$Mab-77 (Figure 5C,D) and 281-m$G_{2a}$-f (Figure 5E,F) could not activate the effector cells. These results indicated that $H_2$Mab-77-m$G_{2a}$-f exhibits superior ADCC activation activity compared with $H_2$Mab-77.

## 3.6 | Antibody-dependent cellular cytotoxicity and CDC by $H_2$Mab-77-m$G_{2a}$-f against breast cancer

We next tested $H_2$Mab-77-m$G_{2a}$-f for ADCC against BT-474 cells (HER2-expressing breast cancer cell line) and MDA-MB-468 cells (HER2-negative breast cancer cell line). As shown in Figure 6A, $H_2$Mab-77-m$G_{2a}$-f demonstrated more ADCC (38.9% cytotoxicity) against BT-474 cells than 281-m$G_{2a}$-f, the control afucosylated mouse $IgG_{2a}$ (5.5% cytotoxicity; $p < 0.001$). In contrast, the ADCC activity of $H_2$Mab-77-m$G_{2a}$-f against MDA-MB-468 cells was 9.3% cytotoxicity, which was comparable to that of 281-m$G_{2a}$-f (10.2% cytotoxicity).

Then, we looked to see whether $H_2$Mab-77-m$G_{2a}$-f had CDC against BT-474 and MDA-MB-468 cells. As indicated in Figure 6B, $H_2$Mab-77-m$G_{2a}$-f induced more cytotoxicity (60.5% cytotoxicity) in BT-474 cells than control afucosylated mouse $IgG_{2a}$ (26.1% cytotoxicity; $p < 0.01$). In contrast, the CDC activity of $H_2$Mab-77-m$G_{2a}$-f was 11.4% cytotoxicity against MDA-MB-468 cells, which was comparable to that of 281-m$G_{2a}$-f (9.3% cytotoxicity). These encouraging findings show that $H_2$Mab-77-m$G_{2a}$-f greatly improves both ADCC and CDC activities against HER2-expressing breast cancer cells.

## 3.7 | Antitumor activities of $H_2$Mab-77-m$G_{2a}$-f in the mouse xenografts of breast tumor cells

Tumor formation was observed on day 7 in 16 mice inoculated with either BT-474 or MDA-MB-468 cells. The mice bearing breast cancer were divided into two groups: one group received treatment with $H_2$Mab-77-m$G_{2a}$-f, and the other group served as the control and received 281-m$G_{2a}$-f. Intraperitoneal injections of $H_2$Mab-77-m$G_{2a}$-f (100 μg) and 281-m$G_{2a}$-f (100 μg) were administered to the respective groups on days 7, 14, and 21 after

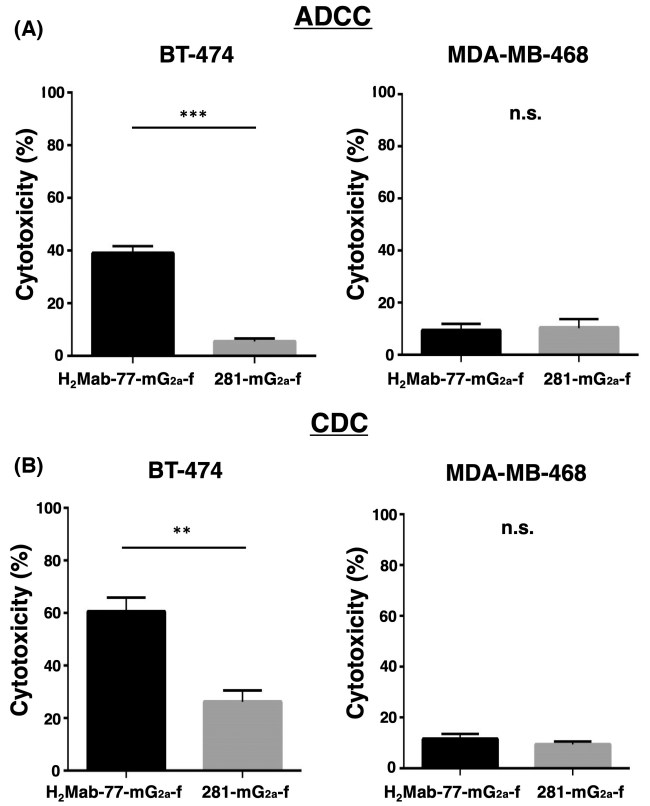


FIGURE 6 Investigation of the antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activities elicited by $H_2$Mab-77-$mG_{2a}$-f. (A) ADCC elicited by $H_2$Mab-77-$mG_{2a}$-f and 281-$mG_{2a}$-f (control antibodies) targeting BT-474 and MDA-MB-468 cells. (B) CDC elicited by $H_2$Mab-77-$mG_{2a}$-f and 281-$mG_{2a}$-f (control antibodies) targeting BT-474 and MDA-MB-468 cells. Values are presented as the mean ± SEM. Statistical significance is indicated by asterisks (***$p < 0.001$, **$p < 0.01$, n.s., not significant, unpaired *t*-test).

cell inoculation. Tumor diameters were measured on days 7, 10, 14, 16, 21, 24, and 28 following cell inoculation. In the BT-474-bearing mice, the group treated with $H_2$Mab-77-$mG_{2a}$-f exhibited significantly less tumor growth on days 24 ($p < 0.01$) and 28 ($p < 0.001$) compared with the group treated with 281-$mG_{2a}$-f (Figure 7A). The reduction in tumor volume achieved by $H_2$Mab-77-$mG_{2a}$-f treatment was 36.6% on day 28. Conversely, there was no difference in tumor growth between the $H_2$Mab-77-$mG_{2a}$-f and 281-$mG_{2a}$-f treatment groups in the MDA-MB-468-bearing mice (Figure 7B). Additionally, the tumor weight of the BT-474-bearing mice treated with $H_2$Mab-77-$mG_{2a}$-f was significantly lower than that of the mice treated with 281-$mG_{2a}$-f (45.1% reduction; $p < 0.01$, Figure 7C). However, no difference in tumor weight on day 28 was observed between the $H_2$Mab-77-$mG_{2a}$-f and 281-$mG_{2a}$-f treatment groups in the MDA-MB-468-bearing mice (Figure 7D). Figure 7E,F show the resected tumors from the $H_2$Mab-77-$mG_{2a}$-f- and 281-$mG_{2a}$-f-treated groups, respectively, on day 28 after inoculation of BT-474 and MDA-MB-468 cells. There were no significant differences in total body weights between the $H_2$Mab-77-$mG_{2a}$-f- and 281-$mG_{2a}$-f-treated groups in both the BT-474- and MDA-MB-468-bearing xenografts (Figure 7G,H). The appearance of mice treated with $H_2$Mab-77-$mG_{2a}$-f and 281-$mG_{2a}$-f on day 28 after cell inoculation is shown in Figure S1. In summary, these results demonstrate the antitumor effects of $H_2$Mab-77-$mG_{2a}$-f administration against HER2-positive breast cancer xenografts.

## 4 | DISCUSSION

Humanized anti-HER2 mAbs like trastuzumab and pertuzumab have helped patients with HER2-positive breast cancer live longer.[19,21,50,51] Aside from monotherapy with these mAbs, combined treatment with trastuzumab and pertuzumab has been shown to enhance outcomes.[52] The membrane-bound p95 generated by the extracellular cleavage of HER2 by metalloproteinase has kinase activity in HER2-overexpressing cells.[27] The inhibition of this cleavage by trastuzumab may be one of the factors suppressing proliferative signals. Furthermore, the antitumor effects of trastuzumab is probably mediated by indirect mechanisms such as ADCC activity.[39] In the future, we will look into the impacts of $H_2$Mab-77-$mG_{2a}$-f on dimer formation and HER2 shedding to better understand the mechanism of the antitumor action by $H_2$Mab-77-$mG_{2a}$-f.

The development of significant acquired resistance is a challenge for the therapeutic use of trastuzumab.[53,54] Concerns have been raised that even strong ADCs, such as T-DM1, may develop resistance owing to diminished antigen binding, poor internalization, lysosomal degradation errors, and accelerated cellular drug clearance by drug-efflux pumps.[55] MUC4, a membrane-associated mucin known to mask membrane proteins, has been shown to inhibit trastuzumab binding to HER2.[56] Furthermore, MUC4 acts as an intramembranous ligand and activator of HER2, resulting in the inhibition of apoptosis in cancer cells.[57]

Antibodies have distinct activities depending on their binding epitopes, similar to trastuzumab and pertuzumab. The FDA-approved margetuximab and trastuzumab have comparable epitopes and binding affinity; however, margetuximab has a greater binding capacity to the ADCC activator FcγRIIIa and a lower affinity for the immune activation inhibitor CD32B.[58–60] Patients with breast cancer who have low-binding FcγRIIIa alleles may also benefit from a combination of margetuximab and anti-HER2 treatments.[61] When used in conjunction with trastuzumab, the HER2-targeted humanized mAb 1E11 inhibits the growth of HER2-expressing gastric tumors by binding to the HER2 domain IV, which does not overlap with trastuzumab.[62] Therefore, one of the primary strategies for combating drug resistance is the development of antibodies with a variety of features, including the binding epitope. $H_2$Mab-19,[63] $H_2$Mab-41,[64] $H_2$Mab-77,[38] $H_2$Mab-119,[65] $H_2$Mab-139,[66] $H_2$Mab-181,[67] $H_2$Mab-214,[68] and $H_2$Mab-250[69] are anti-HER2 mAbs that we have previously established, and some of them were proved to have antitumor effects.[48,63] In our early findings, these mAbs have different epitopes, including HER2 domains I, III, and IV. Trastuzumab and anti-HER2 antibodies

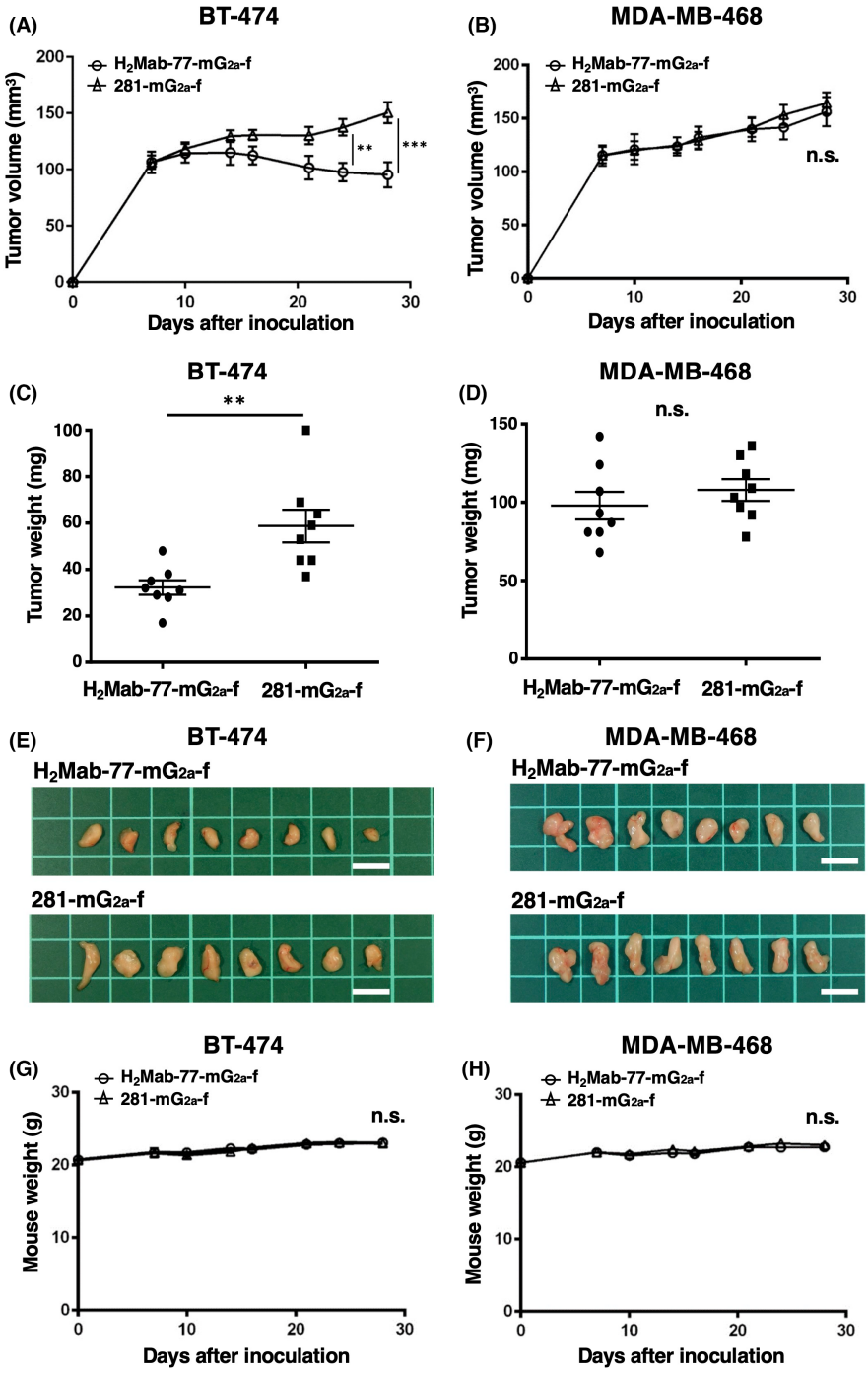


**FIGURE 7** Evaluation of the antitumor activity of $H_2$Mab-77-m$G_{2a}$-f in breast cancer xenograft models. (A, B) BT-474 ($5\times10^6$ cells) (A) and MDA-MB-468 ($5\times10^6$ cells) (B) were subcutaneously transplanted into the left flanks of mice. On day 7 after transplantation, 100 μg of $H_2$Mab-77-m$G_{2a}$-f and 281-m$G_{2a}$-f (control) were intraperitoneally injected into mice. Additional antibody treatments were conducted on days 14 and 21. Tumor diameters were measured on days 7, 10, 14, 16, 21, 24, and 28 after the inoculation of tumor cells. Values are presented as the mean ± SEM. Statistical significance is indicated by asterisks (***$p<0.001$, **$p<0.01$, n.s., not significant, ANOVA, and Sidak's multiple comparisons test). (C, D) Tumors of BT-474 (C) and MDA-MB-468 (D) xenografts were resected from $H_2$Mab-77-m$G_{2a}$-f and 281-m$G_{2a}$-f (control) groups. On day 28, tumor weight was measured from the excised xenografts. Values are presented as the mean ± SEM. Statistical significance is denoted by asterisks (**$p<0.01$, n.s., not significant, unpaired *t*-test). (E, F) Resected tumors of BT-474 (E) and MDA-MB-468 (F) xenografts from $H_2$Mab-77-m$G_{2a}$-f and 281-m$G_{2a}$-f (control) groups on day 28. Scale bar: 1 cm. (G, H) Body weights of mice inoculated with BT-474 (G) and MDA-MB-468 (H) xenografts in $H_2$Mab-77-m$G_{2a}$-f and 281-m$G_{2a}$-f (control) groups were recorded on days 7, 10, 14, 16, 21, 24, and 28. Values are mean ± SEM. No statistical significance is indicated by n.s. (ANOVA and Sidak's multiple comparisons test).

that target several epitopes actually have a stronger antitumor impact than standalone therapies.[70]

$H_2$Mab-77-m$G_{2a}$-f effectively stained HER2 in 14 of 63 cases (22%) of breast cancer (Table S1), showing its use in the immunohistochemical investigation of formalin-fixed paraffin-embedded tumor sections for identifying HER2. However, the information of HER2-positive in the tissue microarray did not necessarily match the result of $H_2$Mab-77-m$G_{2a}$-f staining. Therefore, we speculated that the tissue microarray used in the study may not include the HER2-positive region because HER2-overexpressing tumors are defined by strong and complete immunohistochemistry membranous staining of more than 10% of cells.

Syngeneic mouse models are useful to evaluate the intact antitumor immune system. A transgenic mouse model using the mouse mammary tumor virus promoter-controlled HER2 expression was developed to evaluate mu4D5 (the murine version of trastuzumab).[71] The high level of HER2 expression was achieved in mammary gland epithelium of young virgin founder mice, which resulted in the development of mammary adenocarcinomas in female mice with metastasis.[71] mu4D5 significantly reduced the incidence and growth of mammary adenocarcinomas.[71] Furthermore, the tumors could be propagated as allografts in wild-type mice by implanting a small piece of tumor into the mammary fat pad. Using the allograft model, they also evaluated T-DM1.[72] The modified

$H_2$Mab-77-m$G_{2a}$-f demonstrated ADCC and CDC activities depending on HER2 expression as well as superior antitumor effects in xenograft models of HER2-positive breast cancer (Figures 6 and 7). The abovementioned murine model system is useful to compare the efficacy of $H_2$Mab-77-m$G_{2a}$-f with other HER2-targeted medicines.

Bispecific antibodies targeting HER2×CD3 (ertumaxomab), HER2×CD16, and HER2×HER3 (zenocutuzumab: MCLA-128 and MM-111) are being developed in addition to naive antibodies.[73–78] Recently, progress has been made in the creation of bispecific antibodies that target both the immune checkpoint molecules PD-1/PD-L1 and HER2, with antitumor effects proven in preclinical animals.[79,80] In trastuzumab-resistant cancer models, bispecific antibodies targeting immune checkpoint molecules and HER2 may be more successful than individual mAb treatments. The use of $H_2$Mabs to create bispecific antibodies is another option for increasing anti-HER2 treatment. Furthermore, attention has been drawn to chimeric antigen receptor-T (CAR-T) cell treatment, which possesses both antibody specificity and T cell cytotoxicity.[81–83] While the FDA authorized the first CD19 CAR-T treatment for B-cell lymphoma in 2017, no CAR-T therapeutic targeting HER2 has yet to be produced.[84] In preclinical settings, we previously showed that anti-PDPN CAR-T cells, in which we incorporated the created anti-PDPN mAb into T cells, had strong antitumor efficacy and may release proinflammatory cytokines.[85,86] The application of $H_2$Mab-77 to HER2-targeting CAR-T is expected for future treatment regimen for HER2-positive breast cancer.

## AUTHOR CONTRIBUTIONS

**Tomohiro Tanaka:** Formal analysis; investigation; writing – original draft. **Hiroyuki Suzuki:** Formal analysis; investigation; writing – original draft. **Tomokazu Ohishi:** Investigation. **Mika K. Kaneko:** Methodology. **Yukinari Kato:** Funding acquisition; project administration; writing – review and editing.

## ACKNOWLEDGMENTS

The authors would like to thank Shun-ichi Ohba and Akiko Harakawa (Institute of Microbial Chemistry [BIKAKEN], Numazu, the Microbial Chemistry Research Foundation) for technical assistance with animal experiments.

## FUNDING INFORMATION

This research was supported in part by the Japan Agency for Medical Research and Development (AMED) under grant numbers JP23ama121008 (to Y.K.), JP23am0401013 (to Y.K.), 23bm1123027h0001 (to Y.K.), and JP23ck0106730 (to Y.K.).

## ETHICS STATEMENT

Approval of the research protocol: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: Animal experiments were approved by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (approval no. 2022-056, 2023-001, and 2023-018).

## ORCID

*Hiroyuki Suzuki* https://orcid.org/0000-0003-2646-5132

*Tomokazu Ohishi* https://orcid.org/0000-0002-9039-4474

*Yukinari Kato* https://orcid.org/0000-0001-5385-8201

## REFERENCES

1. Pal SK, Pegram M. Targeting HER2 epitopes. *Semin Oncol*. 2006;33:386-391.
2. Agus DB, Akita RW, Fox WD, et al. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell*. 2002;2:127-137.
3. Garrett TP, McKern NM, Lou M, et al. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol Cell*. 2003;11:495-505.
4. Wallasch C, Weiss FU, Niederfellner G, Jallal B, Issing W, Ullrich A. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *EMBO J*. 1995;14:4267-4275.
5. Alimandi M, Romano A, Curia MC, et al. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene*. 1995;10:1813-1821.
6. Pinkas-Kramarski R, Soussan L, Waterman H, et al. Diversification of neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J*. 1996;15:2452-2467.
7. Cronin KA, Harlan LC, Dodd KW, Abrams JS, Ballard-Barbash R. Population-based estimate of the prevalence of HER-2 positive breast cancer tumors for early stage patients in the US. *Cancer Invest*. 2010;28:963-968.
8. Guarneri V, Barbieri E, Dieci MV, Piacentini F, Conte P. Anti-HER2 neoadjuvant and adjuvant therapies in HER2 positive breast cancer. *Cancer Treat Rev*. 2010;36(Suppl 3):S62-S66.
9. Abd El-Rehim DM, Pinder SE, Paish CE, et al. Expression and co-expression of the members of the epidermal growth factor receptor (EGFR) family in invasive breast carcinoma. *Br J Cancer*. 2004;91:1532-1542.
10. Purdie CA, Baker L, Ashfield A, et al. Increased mortality in HER2 positive, oestrogen receptor positive invasive breast cancer: a population-based study. *Br J Cancer*. 2010;103:475-481.
11. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987;235:177-182.
12. Puglisi F, Fontanella C, Amoroso V, et al. Current challenges in HER2-positive breast cancer. *Crit Rev Oncol Hematol*. 2016;98:211-221.
13. Hayes DF. HER2 and breast cancer–a phenomenal success story. *N Engl J Med*. 2019;381:1284-1286.
14. Arteaga CL, Engelman JA. ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. *Cancer Cell*. 2014;25:282-303.
15. Riecke K, Witzel I. Targeting the human epidermal growth factor receptor family in breast cancer beyond HER2. *Breast Care (Basel)*. 2020;15:579-585.
16. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol*. 2007;25:118-145.
17. Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN. The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. *Oncologist*. 2009;14:320-368.
18. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody

in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol*. 1999;17:2639-2648.
19. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344:783-792.
20. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol*. 2002;20:719-726.
21. Amiri-Kordestani L, Blumenthal GM, Xu QC, et al. FDA approval: ado-trastuzumab emtansine for the treatment of patients with HER2-positive metastatic breast cancer. *Clin Cancer Res*. 2014;20:4436-4441.
22. Nakada T, Sugihara K, Jikoh T, Abe Y, Agatsuma T. The latest research and development into the antibody-drug conjugate, [fam-] trastuzumab deruxtecan (DS-8201a), for HER2 cancer therapy. *Chem Pharm Bull (Tokyo)*. 2019;67:173-185.
23. Zheng Y, Zou J, Sun C, Peng F, Peng C. Ado-tratuzumab emtansine beyond breast cancer: therapeutic role of targeting other HER2-positive cancers. *Front Mol Biosci*. 2023;10:1165781.
24. Hudis CA. Trastuzumab–mechanism of action and use in clinical practice. *N Engl J Med*. 2007;357:39-51.
25. Cho HS, Mason K, Ramyar KX, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin fab. *Nature*. 2003;421:756-760.
26. Shi Y, Fan X, Deng H, et al. Trastuzumab triggers phagocytic killing of high HER2 cancer cells in vitro and in vivo by interaction with Fcγ receptors on macrophages. *J Immunol*. 2015;194:4379-4386.
27. Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J. Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res*. 2001;61:4744-4749.
28. Loo L, Capobianco JA, Wu W, et al. Highly sensitive detection of HER2 extracellular domain in the serum of breast cancer patients by piezoelectric microcantilevers. *Anal Chem*. 2011;83:3392-3397.
29. Junttila TT, Li G, Parsons K, Phillips GL, Sliwkowski MX. Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer. *Breast Cancer Res Treat*. 2011;128:347-356.
30. Ogitani Y, Hagihara K, Oitate M, Naito H, Agatsuma T. Bystander killing effect of DS-8201a, a novel anti-human epidermal growth factor receptor 2 antibody-drug conjugate, in tumors with human epidermal growth factor receptor 2 heterogeneity. *Cancer Sci*. 2016;107:1039-1046.
31. Li BT, Smit EF, Goto Y, et al. Trastuzumab deruxtecan in HER2-mutant non-small-cell lung cancer. *N Engl J Med*. 2022;386:241-251.
32. Shitara K, Bang YJ, Iwasa S, et al. Trastuzumab deruxtecan in previously treated HER2-positive gastric cancer. *N Engl J Med*. 2020;382:2419-2430.
33. Harbeck N, Beckmann MW, Rody A, et al. HER2 dimerization inhibitor pertuzumab–mode of action and clinical data in breast cancer. *Breast Care*. 2013;8:49-55.
34. Barthélémy P, Leblanc J, Goldbarg V, Wendling F, Kurtz JE. Pertuzumab: development beyond breast cancer. *Anticancer Res*. 2014;34:1483-1491.
35. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell*. 2004;5:317-328.
36. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med*. 2000;6:443-446.
37. Hou Y, Nitta H, Li Z. HER2 Intratumoral heterogeneity in breast cancer, an evolving concept. *Cancers (Basel)*. 2023;15:15.
38. Itai S, Fujii Y, Kaneko MK, et al. H2Mab-77 is a sensitive and specific anti-HER2 monoclonal antibody against breast cancer. *Monoclon Antib Immunodiagn Immunother*. 2017;36:143-148.
39. Tsao LC, Force J, Hartman ZC. Mechanisms of therapeutic antitumor monoclonal antibodies. *Cancer Res*. 2021;81:4641-4651.
40. Shinkawa T, Nakamura K, Yamane N, et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem*. 2003;278:3466-3473.
41. Kaneko MK, Ohishi T, Nakamura T, et al. Development of core-fucose-deficient humanized and chimeric anti-human podoplanin antibodies. *Monoclon Antib Immunodiagn Immunother*. 2020;39:167-174.
42. Subramaniam JM, Whiteside G, McKeage K, Croxtall JC. Mogamulizumab: first global approval. *Drugs*. 2012;72:1293-1298.
43. Fujii Y, Kaneko MK, Kato Y. MAP tag: a novel tagging system for protein purification and detection. *Monoclon Antib Immunodiagn Immunother*. 2016;35:293-299.
44. Fujii Y, Kaneko MK, Ogasawara S, et al. Development of RAP tag, a novel tagging system for protein detection and purification. *Monoclon Antib Immunodiagn Immunother*. 2017;36:68-71.
45. Ikota H, Nobusawa S, Arai H, et al. Evaluation of IDH1 status in diffusely infiltrating gliomas by immunohistochemistry using anti-mutant and wild type IDH1 antibodies. *Brain Tumor Pathol*. 2015;32:237-244.
46. Kato Y. Specific monoclonal antibodies against IDH1/2 mutations as diagnostic tools for gliomas. *Brain Tumor Pathol*. 2015;32:3-11.
47. Nanamiya R, Suzuki H, Takei J, et al. Development of monoclonal antibody 281-mG(2a)-f against golden hamster podoplanin. *Monoclon Antib Immunodiagn Immunother*. 2022;41:311-319.
48. Kato Y, Ohishi T, Takei J, et al. An anti-human epidermal growth factor receptor 2 monoclonal antibody H2Mab-19 exerts antitumor activity in mouse colon cancer xenografts. *Monoclon Antib Immunodiagn Immunother*. 2020;39:123-128.
49. Song KH, Trudeau T, Kar A, Borden MA, Gutierrez-Hartmann A. Ultrasound-mediated delivery of siESE complexed with microbubbles attenuates HER2+/− cell line proliferation and tumor growth in rodent models of breast cancer. *Nanotheranostics*. 2019;3:212-222.
50. Valabrega G, Montemurro F, Aglietta M. Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Ann Oncol*. 2007;18:977-984.
51. Amiri-Kordestani L, Wedam S, Zhang L, et al. 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.
52. Swain SM, Kim SB, Cortés J, et al. 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.
53. Derakhshani A, Rezaei Z, Safarpour H, et al. Overcoming trastuzumab resistance in HER2-positive breast cancer using combination therapy. *J Cell Physiol*. 2020;235:3142-3156.
54. Vivekanandhan S, Knutson KL. Resistance to trastuzumab. *Cancers (Basel)*. 2022;14:14.
55. Khoury R, Saleh K, Khalife N, et al. Mechanisms of resistance to antibody-drug conjugates. *Int J Mol Sci*. 2023;24:9674.
56. Nagy P, Friedländer E, Tanner M, et al. Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. *Cancer Res*. 2005;65:473-482.
57. Carraway KL, Perez A, Idris N, et al. Muc4/sialomucin complex, the intramembrane ErbB2 ligand, in cancer and epithelia: to protect and to survive. *Prog Nucleic Acid Res Mol Biol*. 2002;71:149-185.
58. Royce M, Osgood CL, Amatya AK, et al. FDA approval summary: Margetuximab plus chemotherapy for advanced or metastatic HER2-positive breast cancer. *Clin Cancer Res*. 2022;28:1487-1492.
59. Nordstrom JL, Gorlatov S, Zhang W, et al. Anti-tumor activity and toxicokinetics analysis of MGAH22, an anti-HER2 monoclonal

antibody with enhanced Fcγ receptor binding properties. *Breast Cancer Res*. 2011;13:R123.
60. Rugo HS, Im SA, Cardoso F, et al. Efficacy of Margetuximab vs trastuzumab in patients with pretreated ERBB2-positive advanced breast cancer: a phase 3 randomized clinical trial. *JAMA Oncol*. 2021;7:573-584.
61. Mandó P, Rivero SG, Rizzo MM, Pinkasz M, Levy EM. Targeting ADCC: a different approach to HER2 breast cancer in the immunotherapy era. *Breast*. 2021;60:15-25.
62. Ko BK, Lee SY, Lee YH, et al. Combination of novel HER2-targeting antibody 1E11 with trastuzumab shows synergistic antitumor activity in HER2-positive gastric cancer. *Mol Oncol*. 2015;9:398-408.
63. Takei J, Kaneko MK, Ohishi T, Kawada M, Harada H, Kato Y. H2Mab-19, an anti-human epidermal growth factor receptor 2 monoclonal antibody exerts antitumor activity in mouse oral cancer xenografts. *Exp Ther Med*. 2020;20:846-853.
64. Tateyama N, Asano T, Ohishi T, et al. An anti-HER2 monoclonal antibody H2Mab-41 exerts antitumor activities in mouse xenograft model using dog HER2-overexpressed cells. *Monoclon Antib Immunodiagn Immunother*. 2021;40:184-190.
65. Yamada S, Itai S, Nakamura T, et al. Establishment of H(2)Mab-119, an anti-human epidermal growth factor receptor 2 monoclonal antibody, against pancreatic cancer. *Monoclon Antib Immunodiagn Immunother*. 2017;36:287-290.
66. Kaneko MK, Yamada S, Itai S, Kato Y. Development of an anti-HER2 monoclonal antibody H2Mab-139 against colon cancer. *Monoclon Antib Immunodiagn Immunother*. 2018;37:59-62.
67. Takei J, Asano T, Tanaka T, et al. Development of a novel anti-HER2 monoclonal antibody H(2)Mab-181 for gastric cancer. *Monoclon Antib Immunodiagn Immunother*. 2021;40:168-176.
68. Arimori T, Mihara E, Suzuki H, et al. Locally misfolded HER2 expressed on cancer cells is a promising target for development of cancer-specific antibodies. *Cell Press Community Review*. 2023. doi:10.2139/ssrn.4565236. dx.doi.org
69. Suzuki H, Kaneko MK, Kato Y. A cancer-specific monoclonal antibody against HER2 for breast cancers *Preprint*. 2023. doi:10.20944/preprints202309.0906.v1
70. Spiridon CI, Ghetie MA, Uhr J, et al. Targeting multiple her-2 epitopes with monoclonal antibodies results in improved antigrowth activity of a human breast cancer cell line in vitro and in vivo. *Clin Cancer Res*. 2002;8:1720-1730.
71. Finkle D, Quan ZR, Asghari V, et al. HER2-targeted therapy reduces incidence and progression of midlife mammary tumors in female murine mammary tumor virus huHER2-transgenic mice. *Clin Cancer Res*. 2004;10:2499-2511.
72. Lewis Phillips GD, Li G, Dugger DL, et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res*. 2008;68:9280-9290.
73. Turini M, Chames P, Bruhns P, Baty D, Kerfelec B. A FcγRIII-engaging bispecific antibody expands the range of HER2-expressing breast tumors eligible to antibody therapy. *Oncotarget*. 2014;5:5304-5319.
74. Diermeier-Daucher S, Ortmann O, Buchholz S, Brockhoff G. Trifunctional antibody ertumaxomab: non-immunological effects on Her2 receptor activity and downstream signaling. *MAbs*. 2012;4:614-622.
75. Haense N, Atmaca A, Pauligk C, et al. A phase I trial of the trifunctional anti Her2 × anti CD3 antibody ertumaxomab in patients with advanced solid tumors. *BMC Cancer*. 2016;16:420.
76. Schram AM, Odintsov I, Espinosa-Cotton M, et al. Zenocutuzumab, a HER2xHER3 bispecific antibody, is effective therapy for tumors driven by NRG1 gene rearrangements. *Cancer Discov*. 2022;12:1233-1247.
77. Fontana E, Torga G, Fostea R, et al. Sustained tumor regression with Zenocutuzumab, a bispecific antibody targeting human epidermal growth factor receptor 2/human epidermal growth factor receptor 3 signaling, in NRG1 fusion-positive, estrogen receptor-positive breast cancer after progression on a cyclin-dependent kinase 4/6 inhibitor. *JCO Precis Oncol*. 2022;6:e2100446.
78. McDonagh CF, Huhalov A, Harms BD, et al. Antitumor activity of a novel bispecific antibody that targets the ErbB2/ErbB3 oncogenic unit and inhibits heregulin-induced activation of ErbB3. *Mol Cancer Ther*. 2012;11:582-593.
79. Chen YL, Cui Y, Liu X, et al. A bispecific antibody targeting HER2 and PD-L1 inhibits tumor growth with superior efficacy. *J Biol Chem*. 2021;297:101420.
80. Gu CL, Zhu HX, Deng L, et al. Bispecific antibody simultaneously targeting PD1 and HER2 inhibits tumor growth via direct tumor cell killing in combination with PD1/PDL1 blockade and HER2 inhibition. *Acta Pharmacol Sin*. 2022;43:672-680.
81. Boulch M, Cazaux M, Loe-Mie Y, et al. A cross-talk between CAR T cell subsets and the tumor microenvironment is essential for sustained cytotoxic activity. *Sci Immunol*. 2021;6:6.
82. Sureda A, Lugtenburg PJ, Kersten MJ, et al. Cellular therapy in lymphoma. *Hematol Oncol*. 2023.
83. Shin MH, Oh E, Kim Y, et al. Recent advances in CAR-based solid tumor immunotherapy. *Cell*. 2023;12:12.
84. Mullard A. FDA approves first CAR T therapy. *Nat Rev Drug Discov*. 2017;16:669.
85. Shiina S, Ohno M, Ohka F, et al. CAR T cells targeting Podoplanin reduce orthotopic glioblastomas in mouse brains. *Cancer Immunol Res*. 2016;4:259-268.
86. Ishikawa A, Waseda M, Ishii T, Kaneko MK, Kato Y, Kaneko S. Improved anti-solid tumor response by humanized anti-podoplanin chimeric antigen receptor transduced human cytotoxic T cells in an animal model. *Genes Cells*. 2022;27:549-558.

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Tanaka T, Suzuki H, Ohishi T, Kaneko MK, Kato Y. Antitumor activities against breast cancers by an afucosylated anti-HER2 monoclonal antibody $H_2$Mab-77-m$G_{2a}$-f. *Cancer Sci*. 2024;115:298-309. doi:10.1111/cas.16008