A Cancer-Specific Monoclonal Antibody against HER2 for Breast Cancers

Hiroyuki Suzuki *, Mika K Kaneko , Yukinari Kato *

Posted Date: 24 October 2023

doi: 10.20944/preprints202309.0906.v2

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Article

A Cancer-Specific Monoclonal Antibody against HER2 for Breast Cancers

Hiroyuki Suzuki [1,*], Mika K. Kaneko [1] and Yukinari Kato [1,*]

[1] Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan; k.mika@med.tohoku.ac.jp

* Correspondence: hiroyuki.suzuki.b4@tohoku.ac.jp (H.S.); yukinari.kato.e6@tohoku.ac.jp (Y.K.); Tel.: +81-22-717-8207 (H.S. & Y.K.)

Abstract: Overexpression of human epidermal growth factor receptor 2 (HER2) in breast and gastric cancers is an important target for monoclonal antibody (mAb) therapy such as trastuzumab. All therapeutic mAbs, including anti-HER2 mAbs, exhibit adverse effects probably due to the recognition of antigens expressed in normal cells. Therefore, tumor-selective or specific mAbs can be beneficial in reducing the adverse effects. In this study, we provide a strategy for the selection of cancer-specific mAb against HER2. We screened more than 200 of anti-HER2 mAbs obtained by our laboratory and established a novel cancer-specific anti-HER2 antibody, H2Mab-250 (IgG_1, kappa). H2Mab-250 reacted with HER2-positive breast cancer BT-474 and SK-BR-3 cells. Importantly, H2Mab-250 never showed reactivity to non-transformed normal epithelial cells (HaCaT and MCF 10A) and immortalized normal epithelial cells in flow cytometry. In contrast, most anti-HER2 mAbs including H2Mab-119 (IgG_1, kappa) reacted with both cancer and normal epithelial cells. Furthermore, a core-fucose deleted IgG_{2a}-type H2Mab-250 could trigger the antibody-dependent cellular cytotoxicity activity to BT-474, but not to HaCaT cells. Immunohistochemical analysis demonstrated that H2Mab-250 possesses a superior reactivity to the HER2-positive breast cancer section compared to H2Mab-119. More importantly, H2Mab-250 never showed any reactivity to normal tissues. Finally, the epitope mapping demonstrated that H2Mab-250 recognized the domain VI of HER2, and that the Trp614 mainly contributes to the recognition by H2Mab-250. The strategy of selecting cancer-specific mAbs can contribute to the development of novel antibodies and modalities for cancer therapy.

Keywords: HER2; cancer-specific monoclonal antibody; screening; epitope; flow cytometry

1. Introduction

Human epidermal growth factor receptor 2 (HER2) is included in the receptor tyrosine kinase family of human epidermal growth factor receptors. To activate the downstream signaling, HER2 must either form heterodimers with other HER members and their specific ligands or self-assemble into ligand-independent homodimers when overexpressed [1]. The HER2 overexpression is observed in approximately 20% of breast cancers [2] and 20% of gastric cancers [3], which are associated with higher rates of recurrence, poor prognosis, and shorter overall survival. A monoclonal antibody (mAb) against HER2, trastuzumab, exhibited an anti-proliferating effect in vitro and a potent antitumor effect in vivo [4,5]. The addition of trastuzumab to chemotherapy improves objective response rates, progression-free survival, and overall survival in HER2-positive breast cancer patients with metastasis [6]. Trastuzumab has become the standard treatment for HER2-positive breast cancers [7] and HER2-positive gastric cancers [8]. Trastuzumab has been the most effective therapy for HER2-positive breast cancer for more than 20 years [9].

The major adverse effect associated with anti-HER2 therapeutic mAbs is cardiotoxicity, thereby necessitating routine cardiac monitoring in clinics [10]. Furthermore, mice lacking *ErbB2* (ortholog of HER2) displayed embryonic lethal due to the dysfunctions associated with a lack of cardiac trabeculae [11]. Ventricular-restricted *ErbB2*-deficient mice showed the features of dilated cardiomyopathy [12]. These results indicat that HER2 is vital for normal heart development and

 doi:10.20944/preprints202309.0906.v2

homeostasis. Therefore, more selective anti-HER2 mAbs against tumors, which can reduce heart failures are required.

We previously established anti-HER2 mAbs, H2Mab-77 (IgG_1, kappa) [13], H2Mab-119 (IgG_1, kappa) [14], and H2Mab-139 (IgG_1, kappa) [15] by the immunization of HER2 ectodomain produced by cancer cells. We further engineered the mAbs into the mouse IgG_{2a} type (H2Mab-77-mG_{2a}, H2Mab-119-mG_{2a}, and H2Mab-139-mG_{2a}, respectively), and produced the core fucose-deficient types (H2Mab-77-mG_{2a}-f, H2Mab-119-mG_{2a}-f, and H2Mab-139-mG_{2a}-f, respectively) to potentiate the antibody-dependent cellular cytotoxicity (ADCC) and antitumor effect in vivo [16–18]. During the development, we have established a variety of anti-HER2 mAbs. In this study, we screened the anti-HER2 mAbs to select the cancer-specific mAb (CasMab) in accordance with the previously described strategies [19–21], and successfully developed cancer-specific HER2 mAbs.

2. Materials and Methods

2.1. Cell culture

Chinese hamster ovary (CHO)-K1, BT-474, SK-BR-3, MDA-MB-468, MCF 10A, hTERT TIGKs, HBEC3-KT, hTERT-HME1, and RPTEC/TERT1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Human keratinocyte HaCaT was purchased from Cell Lines Service GmbH (Eppelheim, Germany). hTCEpi, hTEC/SVTERT24-B, and HCEC-1CT were purchased from EVERCYTE (Vienna, Austria).

The cDNA of HER2 (wild type; WT) and deletion mutants (dN218, dN342, and dN511) were cloned into the pCAG/nPA16 vector. A HER2 point mutant (W614A) and HER2 WT were cloned into the pCAG/nPA-cRAPMAP vector. CHO-K1 cells were transfected with the above-mentioned vectors using a Neon transfection system (Thermo Fisher Scientific Inc., Waltham, MA). A few days after transfection, PA tag-positive cells were sorted by the cell sorter (SH800; Sony Corp., Tokyo, Japan) using NZ-1, which was originally developed as an anti-human PDPN mAb [22]. Finally, CHO/HER2 and CHO/HER2 (dN218, dN342, and dN511) cell lines were established.

CHO-K1, CHO/HER2 (WT, deletion, and point mutants) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), and BT-474, SK-BR-3, MDA-MB-468, HEK293T, and HaCaT were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc.), 100 units/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Nacalai Tesque, Inc.). Mammary epithelial cell line, MCF 10A was cultured in Mammary Epithelial Cell Basal Medium BulletKit™ (Lonza, Basel, Switzerland) supplemented with 100 ng/ml cholera toxin (Sigma-Aldrich Corp., St. Louis, MO).

Immortalized normal epithelial cell lines were maintained, as follows; hTERT TIGKs, Dermal Cell Basal Medium and Keratinocyte Growth Kit (ATCC); HBEC3-KT, Airway Epithelial Cell Basal Medium and Bronchial Epithelial Cell Growth Kit (ATCC); hTERT-HME1, Mammary Epithelial Cell Basal Medium BulletKit™ without GA-1000 (Lonza); hTCEpi, KGMTM-2 BulletKit™ (Lonza); hTEC/SVTERT24-B, OptiPROTM SFM and GlutaMAX™-I (Thermo Fisher Scientific Inc.); RPTEC/TERT1, DMEM/F-12 and hTERT Immortalized RPTEC Growth Kit with supplement A and B (ATCC); HCEC-1CT, DMEM / M199 (4:1, Thermo Fisher Scientific Inc.), 2 % Cosmic Calf Serum (Cytiva, Tokyo, Japan), 20 ng/ml hEGF (Sigma-Aldrich Corp.), 10 μg/ml insulin (Sigma-Aldrich Corp.), 2 μg/ml apo-transferrin (Sigma-Aldrich Corp.), 5 nM sodium-selenite (Sigma-Aldrich Corp.), 1 μg/ml hydrocortisone (Sigma-Aldrich Corp.).

All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

2.2. CasMab screening

We previously established anti-HER2 mAbs including H2Mab-119 [14] by the immunization of HER2 ectodomain (HER2ec) derived from glioblastoma LN229 or HER2-overexpressed LN229. We finally established 278 clones of hybridoma and performed the flow cytometry-based screening of CasMab using the supernatants.

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2.3. Recombinant mAb production

To generate recombinant H2Mab-250 and H2Mab-119, their V_H cDNAs and the C_H cDNA of mouse IgG_1 were cloned into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The V_L cDNAs and C_L cDNA of the mouse kappa light chain were also cloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation). The vectors were transfected into ExpiCHO-S cells using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.), and Ab-Capcher (ProteNova, Kagawa, Japan) was used to purify the recombinant H2Mab-250 and H2Mab-119.

To generate mouse IgG_{2a}-type H2Mab-250 (H2Mab-250-mG_{2a}), we cloned V_H cDNA of H2Mab-250 and C_H of mouse IgG_{2a} into the pCAG-Ble vector. The mouse kappa light chain vector of H2Mab-250 was described above. To produce the defucosylated form (H2Mab-250-mG_{2a}-f), the vectors were transduced into BINDS-09 (fucosyltransferase 8-knockout ExpiCHO-S) cells using the ExpiCHO Expression System. H2Mab-250-mG_{2a}-f was purified using Ab-Capcher.

2.4. Flow cytometry

Cells were collected using 0.25% trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai Tesque, Inc.). The cells (1×10^5 cells/sample) were treated with H2Mab-250, H2Mab-119, or blocking buffer [control; 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)] for 30 min at 4°C. Next, the cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:2,000; Cell Signaling Technology, Danvers, MA) for 30 min at 4°C. The fluorescence data was collected using EC800 or SA3800 Cell Analyzer (Sony Corp), and the data were analyzed using FlowJo (BD Biosciences, Franklin Lakes, NJ).

2.5. ADCC reporter bioassay

The ADCC reporter bioassay was performed using an ADCC Reporter Bioassay kit (Promega Corporation, Madison, WI), according to the manufacturer's instructions. Target cells (BT-474 and HaCaT, 12,500 cells per well) were cultured in a 96-well white solid plate. H2Mab-250-mG_{2a}-f and trastuzumab (Herceptin; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) 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 co-cultured with antibody-treated target cells at 37°C for 6 h. Luminescence using the Bio-Glo Luciferase Assay System was measured using a GloMax luminometer (Promega Corporation).

2.6. Immunohistochemical analysis

Formalin-fixed paraffin-embedded (FFPE) tissue of HER2-positive breast cancer was obtained from Sendai Medical Center [13]. Informed consent for sample procurement and subsequent data analyses was obtained from the patient or the patient's guardian at Sendai Medical Center. Normal tissues were purchased from BioChain Institute Inc. (Eureka Drive Newark, CA) or Cybrdi Inc. (Frederick, MD). The tissue sections were autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 min. The blocking was performed using SuperBlock T20 (Thermo Fisher Scientific Inc.). The sections were incubated with H2Mab-250 (1, 0.5, or 0.1 μg/mL) and H2Mab-119 (0.5 or 0.1 μg/mL), and then treated with the EnVision+ Kit for mouse (Agilent Technologies, Inc., Santa Clara, CA). The chromogenic reaction was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.). Counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation) and Leica DMD108 (Leica Microsystems GmbH, Wetzlar, Germany) was used to obtain images and examine the sections.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Synthesized peptides covering the HER2 extracellular domain IV and point mutant peptides were synthesized by Sigma-Aldrich Corp. The peptides (10 μg/mL) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc.). Plate washing was performed with

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PBS containing 0.05% (*v*/*v*) Tween 20 (PBST; Nacalai Tesque, Inc.). After blocking with 1% (*w*/*v*) BSA in PBST, H2Mab-250 (10 μg/mL) was added to each well. Then, the wells were further incubated with peroxidase-conjugated anti-mouse immunoglobulins (1:2000 dilution; Agilent Technologies, Inc). Enzymatic reactions were conducted using One-Step Ultra TMB (Thermo Fisher Scientific Inc.). The optical density at 655 nm was measured using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

2.8. Determination of dissociation constant (K_D) via surface plasmon resonance (SPR)

Measurement of K_D between H2Mab-250 and the HER2 peptides was performed using SPR. H2Mab-250 was immobilized on the sensor chip CM5 in accordance with the manufacturer's protocol by Cytiva. Immobilization of H2Mab-250 (10 μg/mL in acetate buffer (pH 4.0); Cytiva) was carried out using an amine coupling reaction. The surface of the flow cell 2 of the sensor chip CM5 was treated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and N-hydroxysuccinimide (NHS), followed by the injection of H2Mab-250. The K_D between H2Mab-250 and the peptides was determined using Biacore X100 (Cytiva). The binding signals were measured using a single cycle kinetics method. The data were analyzed by 1:1 binding kinetics using Biacore X100 evaluation software (Cytiva) to determine the association rate constant (*k*a) and dissociation rate constant (*k*d) and K_D. The affinity constant (K_A) at equilibrium was calculated as $1/K_D$.

3. Results

3.1. Selection of H2Mab-250 possessing the cancer-specific HER2 recognition

We developed 278 clones of anti-HER2 mAbs using recombinant HER2ec derived from glioblastoma LN229 or HER2-overexpressed LN229 as antigens. We further screened the reactivity to HER2-positive breast cancers (BT-474 and SK-BR-3) and non-transformed normal epithelial cells including HaCaT (keratinocyte) and MCF 10A (mammary gland) using flow cytometry (Figure 1A). Although most of the clones including H2Mab-119 (IgG_1, kappa) reacted with both, a clone H2Mab-250 (IgG_1, kappa) reacted with cancer cells, but not with normal epithelial cells. The reactivity was further confirmed after producing the recombinant antibody. As evident from Figure 1B, H2Mab-250 reacted with CHO/HER2, HER2-positive BT-474 and SK-BR-3 cells, but not with triple-negative MDA-MB-468 cells. H2Mab-250 did not react with HaCaT and MCF 10A cells. In contrast, H2Mab-119 (Figure 1B) and a clinically approved anti-HER2 mAb trastuzumab [17] showed similar reactivity to both cancer and normal epithelial cells.

We next investigated the difference in the reactivity to immortalized normal epithelial cells, including hTERT TIGKs (gingiva), HBEC3-KT (lung bronchus), hTERT-HME1 (mammary gland), hTCEpi (corneal), hTEC/SVTERT24-B (thymus), RPTEC/TERT1 (kidney proximal tubule), and HCEC-1CT (colon). H2Mab-250 did not react with those normal cells, while H2Mab-119 was reactive with all immortalized normal epithelial cells (Figure 1C), indicating that H2Mab-250 possesses cancer-specific reactivity against HER2.

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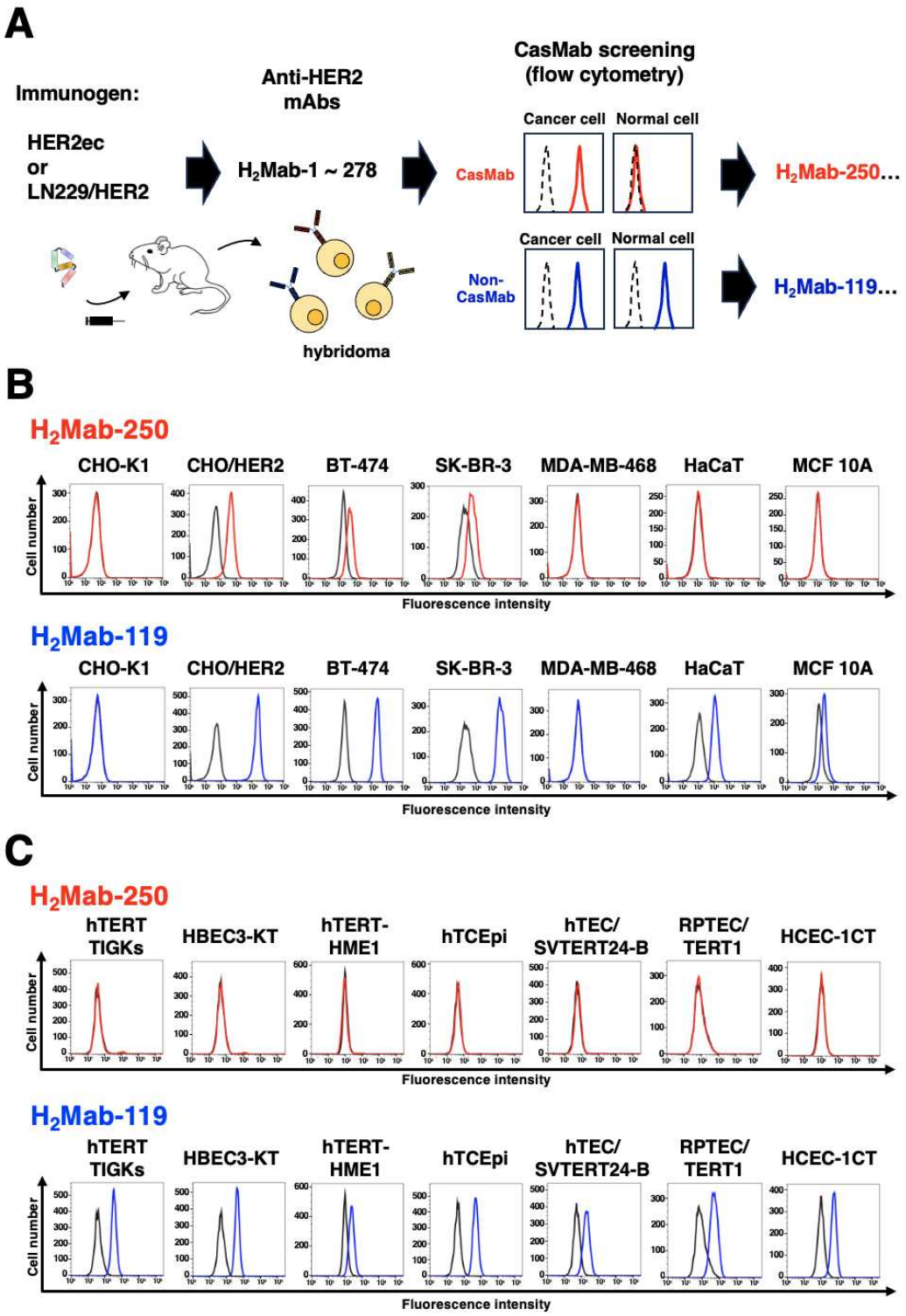

Figure 1. Selection of H2Mab-250, a CasMab against HER2. (A) A scheme of CasMab selection from hybridoma clones. (B) Flow cytometry using H2Mab-250 (Red line) and H2Mab-119 (Blue line) against CHO-K1, CHO/HER2, HER2-positive breast cancers (BT-474 and SK-BR-3), a triple-negative breast cancer (MDA-MB-468), and non-transformed normal epithelial cells (HaCaT and MCF 10A). (C) Flow cytometry using H2Mab-250 (Red line) and H2Mab-119 (Blue line) against immortalized normal epithelial cells including hTERT TIGKs (gingiva), HBEC3-KT (lung bronchus), hTERT-HME1 (mammary gland), hTCEpi (corneal), hTEC/SVTERT24-B (thymus), RPTEC/TERT1 (kidney proximal tubule), and HCEC-1CT (colon). The black line represents the negative control (blocking buffer).

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3.2. The ability of effector cell activation by H2Mab-250 and trastuzumab

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 [23]. We next produced H2Mab-250-mG2a-f, the core-fucose deleted IgG2a version of H2Mab-250 using fucosyltransferase 8-deficient ExpiCHO-S (BINDS-09) cells and examined whether H2Mab-250-mG2a-f could activate ADCC program in the presence of BT-474 and HaCaT cells. To compare the ADCC pathway activation by H2Mab-250-mG2a-f and trastuzumab, we treated BT-474 and HaCaT cells with serially diluted mAbs, and then incubated with effector Jurkat cells, which express the human FcγRIIIa receptor and an NFAT response element driving firefly luciferase. As shown in Figure 2A, H2Mab-250-mG2a-f could activate the effector (EC_{50}: 9.9 μg/ml), but it was less effective than trastuzumab (EC_{50}: 52.7 ng/ml). Importantly, H2Mab-250-mG2a-f did not activate the effector in the presence of HaCaT cells. In contrast, trastuzumab activated the effector with similar EC_{50} (36.2 ng/ml) to BT-474 cells (Figure 2B). These results indicated that H2Mab-250-mG2a-f selectively activates the effector cells against breast cancer cells.

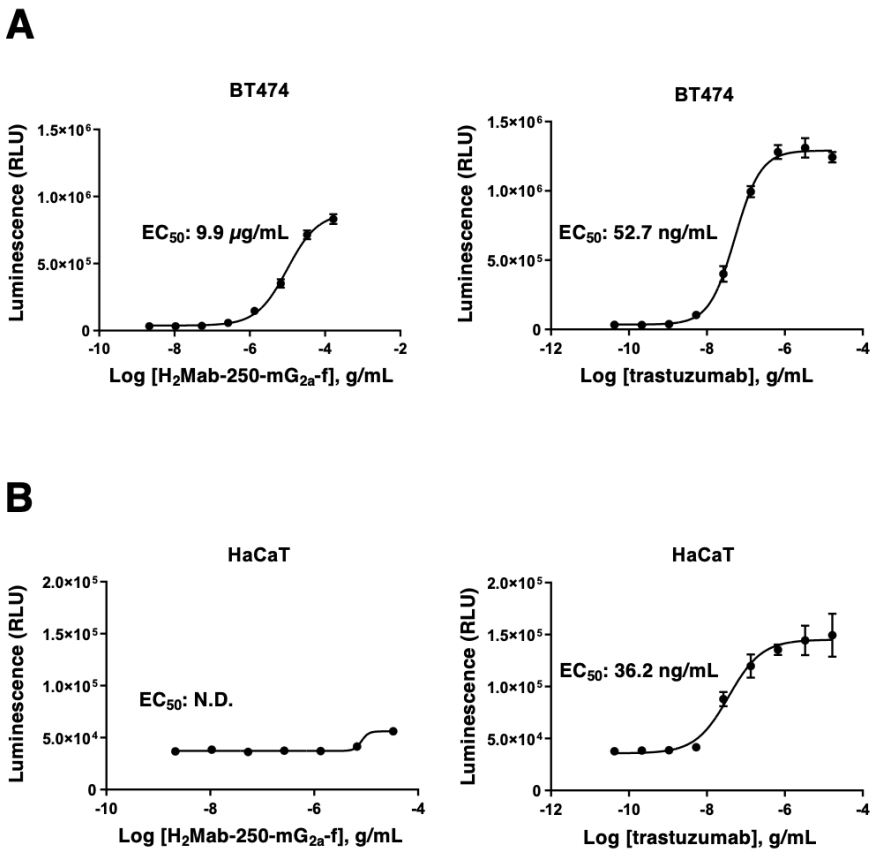

Figure 2. ADCC reporter assay by H2Mab-250-mG2a-f and trastuzumab in the presence of BT-474 and HaCaT cells. Target cells such as BT-474 (A) or HaCaT (B) were cultured in a 96-well white solid plate. H2Mab-250-mG2a-f and trastuzumab were serially diluted and added to the target cells. The engineered Jurkat cells were then added and co-cultured with antibody-treated target cells. Luminescence using the Bio-Glo Luciferase Assay System was measured using a GloMax luminometer. N.D., not determined.

3.3. Immunohistochemical analysis of H2Mab-250 in breast cancer and normal epithelium

Immunohistochemical analysis was performed to examine the reactivity of H2Mab-250 with normal and tumor tissue sections. In contrast to flow cytometry, H2Mab-250 exhibited more potent reactivity to the HER2-positive breast cancer section than H2Mab-119 (Figure 3A). Since all anti-HER2 therapeutic mAbs are associated with cardiotoxicity, a major adverse effect [10], the reactivity of

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H2Mab-250 to a normal heart was further investigated. Even with higher concentrations of H2Mab-250 (1 μg/mL), no reactivity with the normal heart could be detected (Figure 3B). Finally, the reactivity of H2Mab-250 to other normal tissues was investigated. As shown in Figure 3C, no reactivity of H2Mab-250 with any normal tissues, including breast, stomach, lung, colon, kidney, and esophagus could be observed.

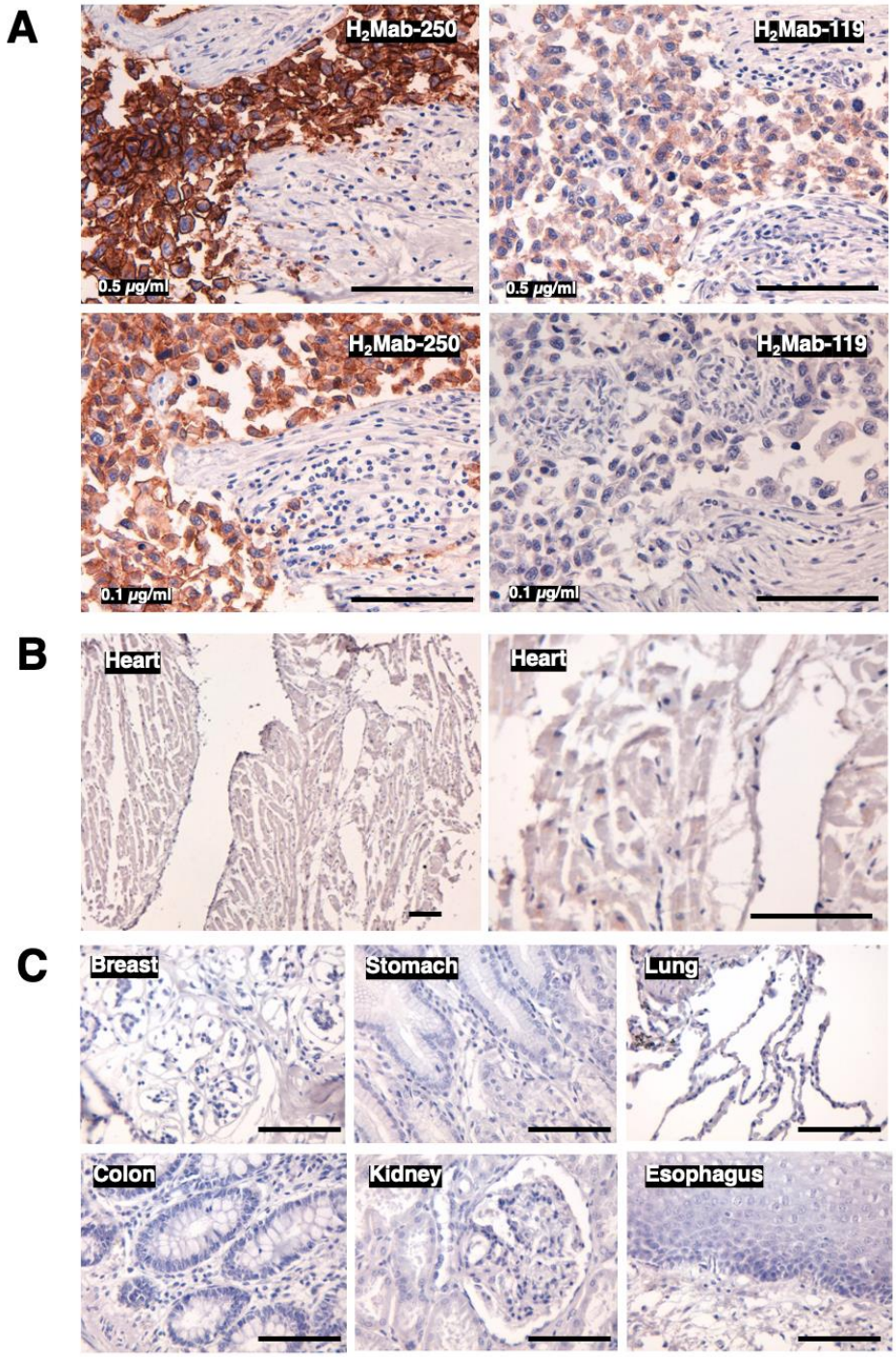

Figure 3. Immunohistochemical analysis of H2Mab-250 in breast cancer and normal epithelium. (A) The HER2-positive breast cancer sections were treated with H2Mab-250 or H2Mab-119 (0.1 or 0.5 μg/mL). (B) A normal heart section was treated with H2Mab-250 (1 μg/mL). (C) Sections of normal breast, stomach, lung, colon, kidney, and esophagus were treated with H2Mab-250 (0.1 μg/mL). The sections were then treated with the Envision+ kit. The chromogenic reaction was performed using DAB, and the sections were counterstained with hematoxylin. Scale bar = 100 μm.

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3.4. Epitope identification for H2Mab-250

To determine the epitope for H2Mab-250, we examined the reactivity to CHO/HER2 (WT) and the N-terminal HER2 deletion mutants (dN218, dN342, and dN511)-expressed CHO-K1 cells (Figure 4A, left). H2Mab-250 reacted with dN218, dN342, dN511, and HER2 (WT). In contrast, H2Mab-119 reacted with only WT, but not with dN218, dN342, and dN511. Since HER2 (WT) and the deletion mutants possess PA16 tag at the N-terminus, all expression on the cell surface could be confirmed by anti-PA16 tag mAb, NZ-1 (Figure 4A, right). These results suggest that H2Mab-250 and H2Mab-119 recognize the domain IV and domain I, respectively.

For further assessment of the H2Mab-250 epitope, ELISA was performed using synthetic peptides that cover the HER2 domain IV. As shown in Figure 4B, H2Mab-250 reacted with HER2 domain IV peptide, amino acids 603–622, 613–632, but not with 593–612, 623–642, and 633–652, indicating that H2Mab-250 recognizes the 613–622 of HER2 domain IV. We further used alanine-substituted peptides of the 603–622 in HER2 domain IV. A potent reduction of the reactivity was observed in the W614A peptide (Figure 4C). We confirmed that the reactivity of H2Mab-250 completely disappeared in CHO/HER2 W614A cells in flow cytometry (Figure 4D).

The dissociation constant (K_D) of H2Mab-250 with the alanine-substituted peptides of HER2 domain IV (603–622) was measured using Biacore X100 (Table 1). The affinity constant (K_A) at equilibrium was calculated as $1/K_D$ (Figure 4E). Compared to the K_A of the 603–622 (WT) peptide, decreased K_A values were observed from the 613–617 region, suggesting that the 613–617 region is involved in the binding to H2Mab-250. A remarkable reduction was measured in the W614A peptide, indicating that Trp614 is mainly involved in the recognition by H2Mab-250.

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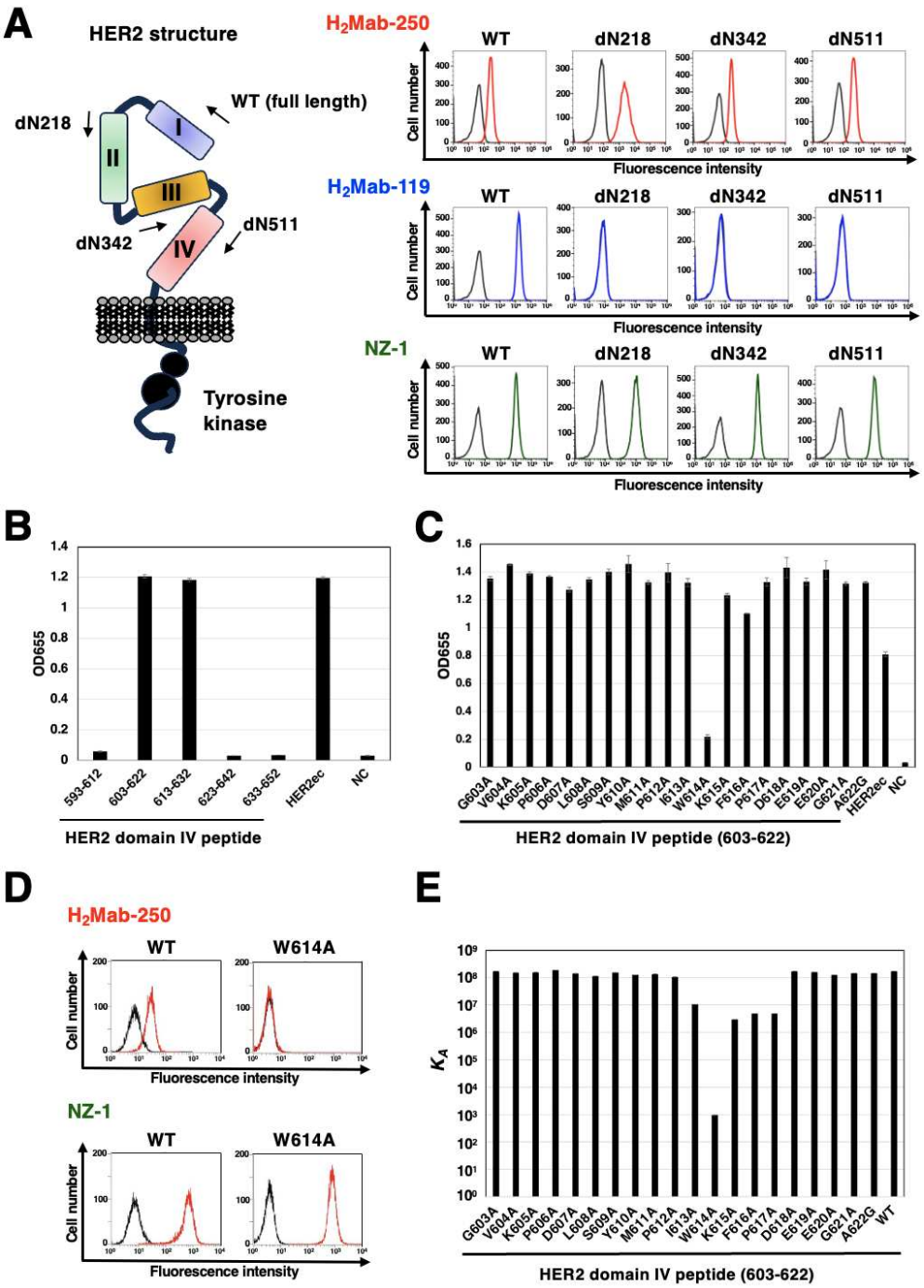

Figure 4. Epitope identification for H2Mab-250. (A) Epitope determination of H2Mab-250 and H2Mab-119 using flow cytometry. The schematic representation of HER2 and the deletion mutants (left). Flow cytometry using H2Mab-250 (Red line) and H2Mab-119 (Blue line) against CHO/HER2 (WT and deletion mutants). The cell surface expression was confirmed by an anti-PA tag mAb, NZ-1 (Green). The black line represents the negative control (blocking buffer). (B and C) Determination of H2Mab-250 epitope by ELISA. Five synthesized peptides that cover the HER2 domain IV (B), alanine-substituted peptides of HER2 domain IV (603–622) (C), HER2ec, or buffer control (NC) were immobilized on immunoplates. The plates were incubated with H2Mab-250, followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins. Optical density was measured at 655 nm. (D) Flow cytometry using H2Mab-250 against CHO/HER2 (WT and W614A). The cell surface expression was confirmed by an anti-PA tag mAb, NZ-1. The black line represents the negative control (blocking buffer). (E) Surface plasmon resonance analysis between H2Mab-250 and HER2 domain IV (603–622) peptides. The affinity constant (K_A) at equilibrium was calculated as $1/K_D$.

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Table 1. Identification of H2Mab-250 epitope using point mutants by Biacore.

Peptide	*Sequence*	*KD (M)*
603-622 (WT)	GVKPDLSYMPIWKFPDEEGA	5.8×10^{-9}
G603A	AVKPDLSYMPIWKFPDEEGA	5.9×10^{-9}
V604A	GAKPDLSYMPIWKFPDEEGA	6.5×10^{-9}
K605A	GVAPDLSYMPIWKFPDEEGA	6.5×10^{-9}
P606A	GVKADLSYMPIWKFPDEEGA	5.3×10^{-9}
D607A	GVKPALSYMPIWKFPDEEGA	7.1×10^{-9}
L608A	GVKPDASYMPIWKFPDEEGA	8.8×10^{-9}
S609A	GVKPDLAYMPIWKFPDEEGA	6.5×10^{-9}
Y610A	GVKPDLSAMPIWKFPDEEGA	7.9×10^{-9}
M611A	GVKPDLSYAPIWKFPDEEGA	7.5×10^{-9}
P612A	GVKPDLSYMAIWKFPDEEGA	9.5×10^{-9}
I613A	GVKPDLSYMPAWKFPDEEGA	9.4×10^{-8}
W614A	GVKPDLSYMPIAKFPDEEGA	1.1×10^{-3}
K615A	GVKPDLSYMPIWAFPDEEGA	3.4×10^{-7}
F616A	GVKPDLSYMPIWKAPDEEGA	2.0×10^{-7}
P617A	GVKPDLSYMPIWKFADEEGA	2.1×10^{-7}
D618A	GVKPDLSYMPIWKFPAEEGA	5.8×10^{-9}
E619A	GVKPDLSYMPIWKFPDAEGA	6.3×10^{-9}
E620A	GVKPDLSYMPIWKFPDEAGA	8.0×10^{-9}
G621A	GVKPDLSYMPIWKFPDEEAA	6.9×10^{-9}
A622G	GVKPDLSYMPIWKFPDEEGG	6.9×10^{-9}

4. Discussion

In this study, we developed a cancer-specific mAb targeting HER2. H2Mab-250 can recognize breast cancer cells, but not normal cells in flow cytometry (Figure 1) and immunohistochemistry (Figure 3). H2Mab-250-mG2a-f could activate ADCC program against breast cancer cells, but not against normal epithelial cells (Figure 2). We also identified the H2Mab-250 epitope sequence (613-IWKFP-617) by SPR analysis (Figure 4). Furthermore, no reaction was observed between H2Mab-250 and CHO/HER2 W614A in flow cytometry (Figure 4), indicating that Trp614 plays a central role in recognition by H2Mab-250 in living cells. Although H2Mab-250 possesses a high affinity to epitope-containing peptide (603–622) in SPR analysis, the recognition in flow cytometry was low compared with that of H2Mab-119 (Figure 4). In contrast, H2Mab-250 exhibited a higher reactivity than H2Mab-119 in the immunohistochemical analysis of breast cancer (Figure 3). This can be attributed to the possibility that the epitope sequence is partially exposed in cancer cells, but not in normal cells in living cells. The mechanism of recognition by H2Mab-250 should be further investigated in future studies. Furthermore, the strategy of CasMab selection can contribute to the development of novel mAbs against a variety of antigens.

For the clinical treatment of metastatic breast cancer, trastuzumab is administered in patients with HER2-overexpressing tumors, which are defined by strong and complete IHC membranous staining of more than 10% of cells (IHC 3+) and/or in situ hybridization (ISH)-amplified. Furthermore, trastuzumab-based antibody-drug conjugates (ADCs) such as trastuzumab-deruxtecan (T-DXd) have been evaluated in various clinical trials. Based on the studies, T-DXd has been approved in not only HER2-positive breast cancer [24,25], but also HER2-mutant lung cancer [26] and HER2-low (IHC 1+ or IHC 2+ / ISH-non-amplified) advanced breast cancer [27]. A significant number of patients can benefit from T-DXd therapy, since approximately half of all breast cancers are classifiable as HER2-low [28]. Meanwhile, cardiotoxicity is the most significant toxicity associated with T-DXd [29]. Further studies are essential to evaluate in vivo toxicities of H2Mab-250.

H2Mab-250-mG2a-f could trigger the ADCC activity to BT-474 selectively (Figure 2). Although the effect of H2Mab-250-mG2a-f is lower than that of trastuzumab, we should consider that effector Jurkat cells express human FcγRIIIa receptor. In contrast, H2Mab-250 exhibited a superior reactivity

to HER2-positive breast cancer section in immunohistochemistry (Figure 3). Therefore, the evaluation of in vivo efficacy against tumor is essential. Furthermore, chimeric antigen receptor (CAR)-T cell therapy against HER2 has been evaluated in clinical studies [28]. It would be worthwhile to investigate the cancer specificity of H_2Mab-250 scFv and the efficacy of CAR-T against HER2-positive tumors, although the optimal epitope for HER2-targeting CAR-T has not yet been evaluated.

Author Contributions: H.S. and M.K.K. performed the experiments. M.K.K. and Y.K. designed the experiments. H.S. and Y.K. analyzed the data. H.S. and Y.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported in part by Japan Agency for Medical Research and Development (AMED) under Grant Numbers: JP23ama121008 (to Y.K.), JP23am0401013 (to Y.K.), 23bm1123027h0001 (to Y.K.), JP23ck0106730 (to Y.K.), JP18am0301010 (to Y.K.), and JP21am0101078 (to Y.K.).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article.

Conflicts of Interest: The authors declare no conflict of interest.

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