MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 40, Number 6, 2021

DOI: 10.1089/mab.2021.0029

Short Communications

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Epitope Mapping of an Anti-HER2 Monoclonal Antibody (H_2Mab-181) Using Enzyme-Linked Immunosorbent Assay

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Human epidermal growth factor receptor 2 (HER2) is a type I transmembrane 185 kDa protein expressed in various types of normal or cancer cells. Overexpression of HER2 is found in many cancers and is related to cell proliferation, differentiation, and migration. We recently developed a novel anti-HER2 monoclonal antibody, H_2Mab-181, by immunizing mice with the purified recombinant extracellular domain of HER2. H_2Mab-181 can specifically and sensitively detect HER2 not only in flow cytometry and Western blotting for gastric cancer cell lines, but also in immunohistochemical analyses for gastric cancer tissues. In this study, we analyzed the binding epitope of H_2Mab-181 to HER2 using enzyme-linked immunosorbent assay (ELISA). Results showed that the H_2Mab-181 epitope was determined to be Gly383, Asp384, Ala386, Asn388, and Pro391 by ELISA.

Keywords: HER2, monoclonal antibody, epitope mapping, ELISA

Introduction

Epidermal growth factor receptor (EGFR) family protein is a type I transmembrane glycoprotein composed of three regions: extracellular, single transmembrane, and intracellular regions.[1,2] The extracellular region contains four domains, namely, I, II, III, and IV domains, and the intracellular region contains a kinase domain.[3] The EGFR family comprises four closely related members, namely, EGFR (HER1/ErbB1), human epidermal growth factor receptor 2 (HER2; ErbB2/Neu), HER3 (ErbB3), and HER4 (ErbB4).[4] The EGFR family members transduce extracellular signals into intracellular signaling pathways through activation of the tyrosine kinase domain. These receptor tyrosine kinases play a critical role in the regulation of cell proliferation, survival, migration, and differentiation.[1] The ligand binding to the extracellular domain promotes the formation of homo- or heterodimers between the EGFR family receptors.[5] However, the ligand of HER2 has not been identified among the HER family and is represented as an activated structure without ligand binding.[6–8] Homodimers or heterodimers are formed between HER2 and other EGFR family members.[6] Dimers including HER2 have a higher tyrosine kinase activity. Among them, HER2/HER3 heterodimer has the greatest transforming and mitogenic signaling ability.[9,10]

HER2 is expressed in various cell types, excluding the cells of hematopoietic origin.[11] Overexpression and mutation of HER2 have been reported in many cancers, including breast,[12,13] ovarian,[14] pancreatic,[15] lung,[16] colorectal,[17] and gastric cancers.[18] In HER2-positive breast cancer patients, this oncogene overexpression is associated with poor prognosis and aggressive tumor phenotypes.[12,13]

Recently, trastuzumab and pertuzumab, which are anti-HER2 monoclonal antibodies (mAbs), have been widely used. Trastuzumab, a humanized murine mAb, was the first approved anti-HER2 mAb drug for breast cancer in 1998 by the U.S. Food and Drug Administration (FDA).[19–21] The use of trastuzumab in gastric cancers began in 2010.[22] Pertuzumab is a fully humanized mAb, and it was approved in 2012 by FDA for breast cancer combination therapy with trastuzumab and docetaxel.[23,24] Trastuzumab binds to

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domain IV of HER2 that contains the binding pocket for the extended domain II loop of the inactive states of the EGFR and HER3. The interaction of trastuzumab is mediated by three loop regions on HER2, formed by residues 557–561, 570–573, and 593–603.[25] Pertuzumab binds the center of domain II of HER2, and pertuzumab binding should block the region necessary for dimerization with the other EGFR family.[26] The difference in the epitope region caused different inhibition mechanisms and antitumor effects.Trastuzumab inhibits the growth of tumors highly overexpressing HER2. In contrast, pertuzumab inhibits tumor growth of both low- and high-expressing HER2 levels.[27] Therefore, identification of the epitope is important to develop the mAb drugs.

Previously, we developed a novel anti-HER2 mAb, H_2Mab-181 (IgG_1, kappa),[28] through the immunization of mice with the purified recombinant protein for the HER2 extracellular domain including domains I–IV.[29-37] H_2Mab-181 can specifically and sensitively detect HER2 in flow cytometry and Western blotting applications in gastric cancer cell lines. It can also be used in immunohistochemical analysis of gastric cancer tissues. In this study, we investigated the epitope of H_2Mab-181 for HER2 using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Peptides

HER2 (Accession No.: M11730) peptides, including 62 deletion mutants (Fig. 1) and 20 alanine-substituted peptides (Table 1), were synthesized by utilizing PEPScreen (Sigma-Aldrich Corp., St. Louis, MO, USA).

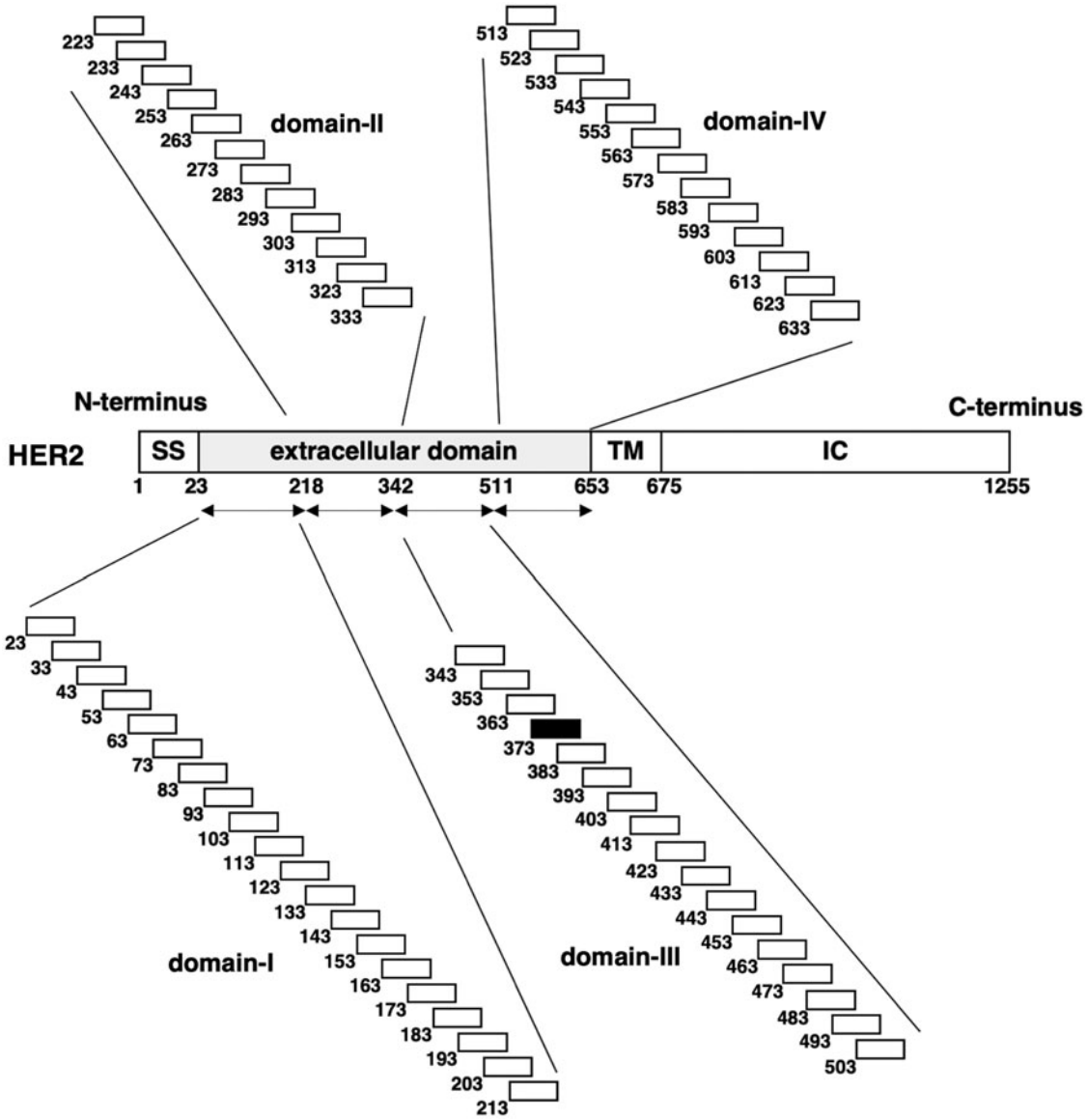

FIG. 1. Epitope mapping of H_2Mab-181 using deletion mutants of HER2 by ELISA. The synthesized peptides were immobilized on immunoplates. The plates were incubated with H_2Mab-181 (1 μg/mL), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins. HER2 and synthesized peptides were illustrated. A *black bar* indicate the peptide detected by H_2Mab-181. ELISA, enzyme-linked immunosorbent assay; HER2, human epidermal growth factor receptor 2; IC, intracellular domain; SS, signal sequence; TM, transmembrane domain.

Table 1. Identification of H_2Mab-181 Epitope Using Enzyme-Linked Immunosorbent Assay

Peptide	*Sequence*	*H_2Mab-181*
373–392	SLAFLPESFDGDPASNTAPL	+
S373A	ALAFLPESFDGDPASNTAPL	+
L374A	SAAFLPESFDGDPASNTAPL	+
A375G	SLGFLPESFDGDPASNTAPL	+
F376A	SLAALPESFDGDPASNTAPL	+
L377A	SLAFAPESFDGDPASNTAPL	+
P378A	SLAFLAESFDGDPASNTAPL	+
E379A	SLAFLPASFDGDPASNTAPL	+
S380A	SLAFLPEAFDGDPASNTAPL	+
F381A	SLAFLPESADGDPASNTAPL	+
D382A	SLAFLPESFAGDPASNTAPL	+
G383A	SLAFLPESFDADPASNTAPL	−
D384A	SLAFLPESFDGAPASNTAPL	−
P385A	SLAFLPESFDGDAASNTAPL	+
A386G	SLAFLPESFDGDPGSNTAPL	−
S387A	SLAFLPESFDGDPAANTAPL	+
N388A	SLAFLPESFDGDPASATAPL	−
T389A	SLAFLPESFDGDPASNAAPL	+
A390G	SLAFLPESFDGDPASNTGPL	+
P391A	SLAFLPESFDGDPASNTAAL	−
L392A	SLAFLPESFDGDPASNTAPA	+

+, OD655 ≥ 0.1; −, OD655 < 0.1.

Enzyme-linked immunosorbent assay (ELISA)

Synthesized HER2 peptides using PEPScreen (Table 1) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a concentration of 10 μg/mL, respectively, for 30 minutes at 37°C. After washing with phosphate-buffered saline containing 0.05% Tween20 (PBST), wells were blocked with 1% bovine serum albumin-containing PBST for 30 minutes at 37°C. The plates were then incubated with H_2Mab-181 (1 μg/mL), followed by a 1:2000 dilution of peroxidase-conjugated anti-mouse immunoglobulins (Agilent Technologies, Inc., Santa Clara, CA, USA). Enzymatic reactions were performed using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc., Kyoto, Japan). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). These reactions were performed with a total sample volume of 50–100 μL.

Results

Epitope mapping by ELISA using deletion mutants

Determination of binding epitope with deletion mutants using PEPScreen is a basic method for determining the linear epitope of mAbs. Furtheremore, alanine scanning using PEPScreen is also very easy to exactly determine the binding epitope.

We synthesized 62 HER2 peptides of 20 amino acids (aa) length (Fig. 1) and performed ELISA. Results showed that H_2Mab-181 reacted with the 373–392 aa ($_{373}$-SLAFLPESFD GDPASNTAPL-$_{392}$) sequence (Fig. 1), indicating that the H_2Mab-181 epitope is located in domain III of HER2.

Epitope mapping by ELISA using alanine-substituted peptides

Next, we synthesized 20 alanine-substituted HER2 peptides of 373–392 aa (Table 1). H_2Mab-181 reacted with S373A, L374A, A375G, F376A, L377A, P378A, E379A, S380A, F381A, D382A, P385A, S387A, T389A, A390G, L392A, and wild-type 373–392 aa, but not with G383A, D384A, A386G, N388A, and P391A (Table 1 and Fig. 2A), indicating that Gly383, Asp384, Ala386, Asn388, and Pro391 are the critical epitope of H_2Mab-181 (Fig. 2B).

Although we also tried to determine the binding epitope of H_2Mab-181 using surface plasmon resonance (SPR), those data were immature probably because the binding affinity is low for the SPR analysis (data not shown). We should further optimize the condition of SPR for the biological characterization in a future study.

Discussion

Domains I and III of HER2 are named as ligand-binding domains although there is no ligand for HER2; domain II mediates the dimer formation, and domain IV contains the binding pocket for the extended domain II loop of the inactive states EGFR and HER3.[38] Trastuzumab binds to domain IV and induces antibody-dependent cell-mediated cytotoxicity.[25] Meanwhile, pertuzumab binds to domain II and inhibits the ligand-induced dimerization.[26] Previously, some mAbs-targeted domain III of HER2 were reported.[39,40] A novel anti-HER2 mAb (hHERmAb-F0178C1) effectively blocked HER2/HER3 heterodimerization and signaling. Its combined use with pertuzumab had a synergistic effect.[39] Another novel anti-HER2 mAb (3E10) inhibited HER2 heterodimerization with EGFR and HER3 through a different mechanism from trastuzumab and pertuzumab.[40] Furthermore, synergistic inhibition of HER2 heterodimerization and signaling was observed with 3E10 in combination with either trastuzumab or pertuzumab.[40] These studies show that targeting domain III of HER2 may be a new target for cancer treatment. Moreover, we showed that H_2Mab-181 is suitable for various applications, including flow cytometry, Western blotting, and immunohistochemical analyses for gastric cancer.[28] Therefore, we will study the antitumor effect of H_2Mab-181 against gastric cancer in the future.

Authors' Contributions

T.A., J.T., T.N., and H.S. performed experiments; M.K.K. designed the experiments; and T.A. and Y.K. wrote the article.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported, in part, by Japan Agency for Medical Research and Development (AMED) under

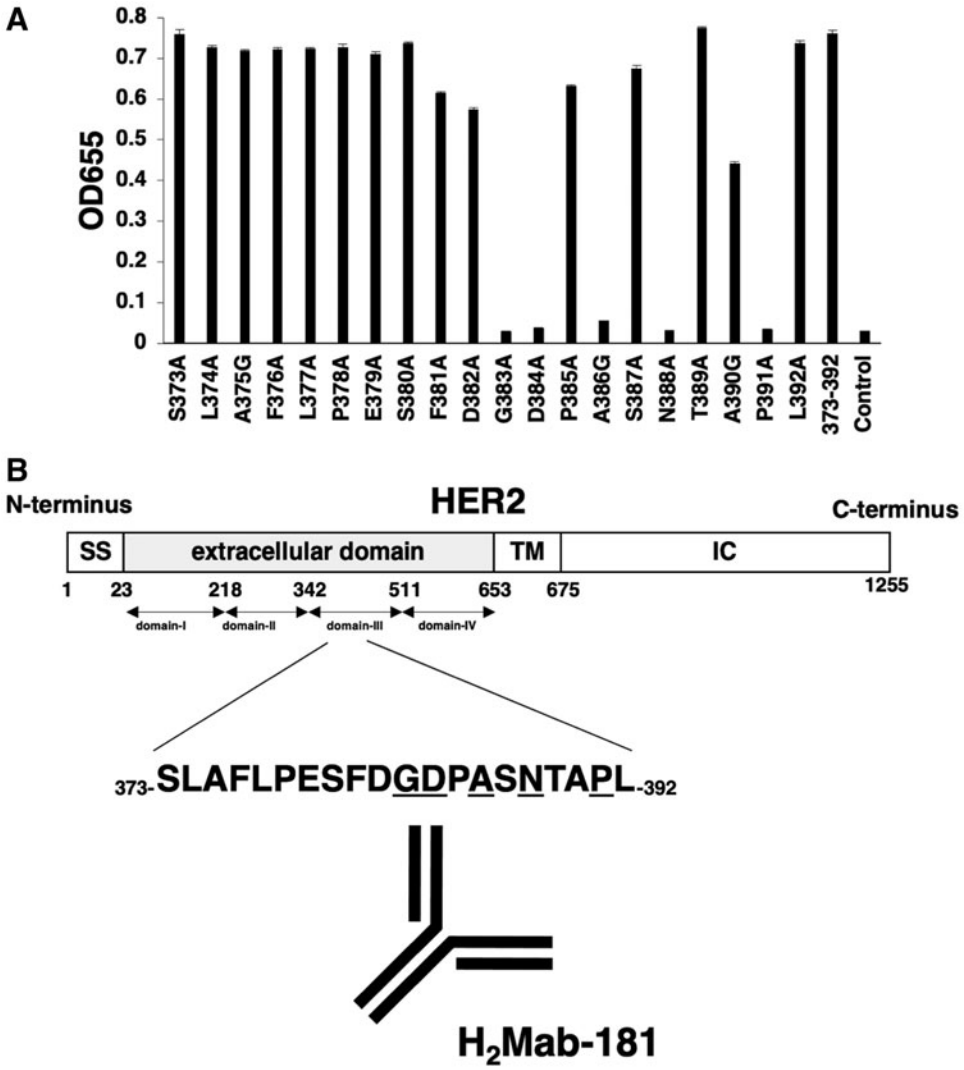

FIG. 2. **(A)** Determination of H_2Mab-181 epitope on HER2 by ELISA using point mutants. The 373–392 peptide (positive control) and synthesized alanine-substituted peptides were immobilized on immunoplates. The plates were incubated with H_2Mab-181 (1 μg/mL), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins. The optical density was measured at 655 nm. **(B)** Schematic illustration of the epitope recognized by H_2Mab-181 using ELISA. Underlined amino acids indicate the critical epitope of H_2Mab-181 determined using ELISA.

Grant Nos.: JP21am0401013 (to Y.K.) and JP21am0101078 (to Y.K.), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) Grant Nos. 21K15523 (to T.A.), 21K07168 (to M.K.K.), and 19K07705 (to Y.K.).

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Received: June 5, 2021
Accepted: September 25, 2021