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Epitope Mapping of an Anti-EpCAM Monoclonal Antibody (EpMab-37) Using the Alanine Scanning Method

Guanjie Li,[1,*] Hiroyuki Suzuki,[1,*] Tomohiro Tanaka,[2] Teizo Asano,[2] Takeo Yoshikawa,[3] Mika K. Kaneko,[2] and Yukinari Kato[1–3]

The epithelial cell adhesion molecule (EpCAM) is a type I transmembrane glycoprotein, and plays critical roles in cell adhesion, proliferation, and tumorigenesis. EpCAM has been considered as a promising target for tumor diagnosis and therapy. Anti-EpCAM monoclonal antibodies (mAbs) have been developed for EpCAM-overexpressed tumors, and several clinical trials have demonstrated promising outcomes. We previously established an anti-EpCAM mAb, EpMab-37 (mouse IgG_1, kappa), using the Cell-Based Immunization and Screening method. EpMab-37 was revealed to recognize the conformational epitope of EpCAM. In this study, we determined the critical epitope of EpMab-37 by flow cytometry using the 1 × alanine scanning (1 × Ala-scan) and the 2 × alanine scanning (2 × Ala-scan) method. We first performed flow cytometry by 1 × Ala-scan using one alanine (or glycine)-substituted EpCAM mutants, which were expressed on Chinese hamster ovary-K1 cells, and found that the EpMab-37 did not recognize the R163A mutant of EpCAM. We next performed flow cytometry by 2 × Ala-scan using two alanine (or glycine) residues-substituted EpCAM mutants, and confirmed that EpMab-37 did not recognize R163A-including mutants of EpCAM. The results indicated that the critical binding epitope of EpMab-37 includes Arg163 of EpCAM.

Keywords: EpCAM, epitope mapping, monoclonal antibody, flow cytometry, alanine scanning

Introduction

Epithelial cell adhesion molecule (EpCAM) is a unique type I transmembrane glycoprotein and is expressed on the basolateral membrane of epithelial cells.[1] EpCAM-mediated intercellular adhesion is essential for the maintenance of the epithelial integrity.[2] Furthermore, overexpression of EpCAM is observed in breast, prostate, gastrointestinal, and colorectal cancers.[3,4] EpCAM was the first identified as human tumor antigen.[3] After cleavage of the EpCAM intracellular domain, it serves as a transcriptional cofactor with β-catenin and regulates the transcriptional target genes, which is involved in cell proliferation, survival, and tumorigenesis.[5]

Therefore, EpCAM has been considered as a target of the monoclonal antibody (mAb) therapies. Several mAbs including adecatumumab,[6] edrecolomab,[7,8] oportuzumab monatox,[9] and catumaxomab[10,11] have been clinically evaluated.

EpCAM is an important marker to isolate circulating tumor cells (CTCs), which play critical roles in tumor progression and metastasis.[12] CTCs provide important prognostic information as an indicator of micrometastasis, and determine the response to some cancer therapies.[13] The U.S. Food and Drug Administration confirmed the clinical importance of CTCs, and approved of CellSearch®, which uses an anti-EpCAM mAb to collect EpCAM-positive CTCs from blood samples.[14]

The EpCAM N-terminal domain (24–63 amino acids [aa]; ND) contains EGF-like domain and is known to possess the high accessibility and antigenicity.[15] Majority of anti-EpCAM mAbs including HEA125 (used in CellSearch), 17-1A (edrecolomab), C215 (used in catumaxomab), and MOC31 (used in oportuzumab) recognize the EpCAM ND.[16,17] In contrast, the anti-EpCAM mAbs, which recognize the thyroglobulin-like domain (64–138 aa; TY) or the extracellular C-terminal domain (139–265 aa; CD), are

Departments of [1]Molecular Pharmacology, [2]Antibody Drug Development, and [3]Pharmacology, Tohoku University Graduate School of Medicine, Sendai, Japan.
*These authors contributed equally to this study.

rare.[16] Among clinically tested mAbs, MT201 (adecatumumab) recognizes the EpCAM CD.[18] Therefore, the detailed determination of mAbs epitope could contribute the understanding of the property of mAbs.

We previously established an anti-EpCAM mAb, EpMab-37 (mouse IgG_1, kappa), by the Cell-Based Immunization and Screening (CBIS) method.[19] To clarify further characteristics of EpMab-37, we performed epitope mapping by flow cytometry using the 1×alanine scanning (1×Ala-scan) method and the 2×alanine scanning (2×Ala-scan) method.

Materials and Methods

Plasmids

EpCAM cDNAs plus an N-terminal PA tag that is recognized by the anti-PA tag mAb (NZ-1)[20–23] were subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). An N-terminal PA-tagged EpCAM[19] and 1×and 2×alanine substituted mutants were produced using a HotStar HiFidelity Polymerase Kit (Qiagen, Inc., Hilden, Germany), and subcloned into the pCAG-Ble vector using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan).

Cell lines

Chinese hamster ovary-K1 (CHO-K1) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Mutant EpCAM-overexpressed CHO-K1 cells were established by transfecting pCAG/EpCAM mutant vectors into CHO-K1 cells using the Neon Transfection System (Thermo Fisher Scientific, Inc., Waltham, MA) as described previously.[24,25] All cells were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan). The media were supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Flow cytometry

Cells were harvested after brief exposure to 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin in phosphate-buffered saline, cells were treated with 1 μg/mL of recombinant EpMab-37[19] and 1 μg/mL of NZ-1 for 30 min at 4°C, and then with Alexa Fluor 488-conjugated anti-mouse or anti-rat IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Results

Epitope mapping of EpMab-37 with alanine (glycine)-substituted EpCAM mutants

We previously established EpMab-37 by the CBIS method. We further constructed the deletion mutants of EpCAM, and found that the EpMab-37 epitope exists in the EpCAM CD (from 144 to 164 aa).[19] To reveal the detailed binding epitope of EpMab-37, we first established several cell lines, which express one-alanine (or glycine)-substituted mutants of EpCAM between 144 and 173 aa. The epitope mapping using these one-alanine (or glycine)-substituted mutants is called as 1×Ala-scan method (Fig. 1).

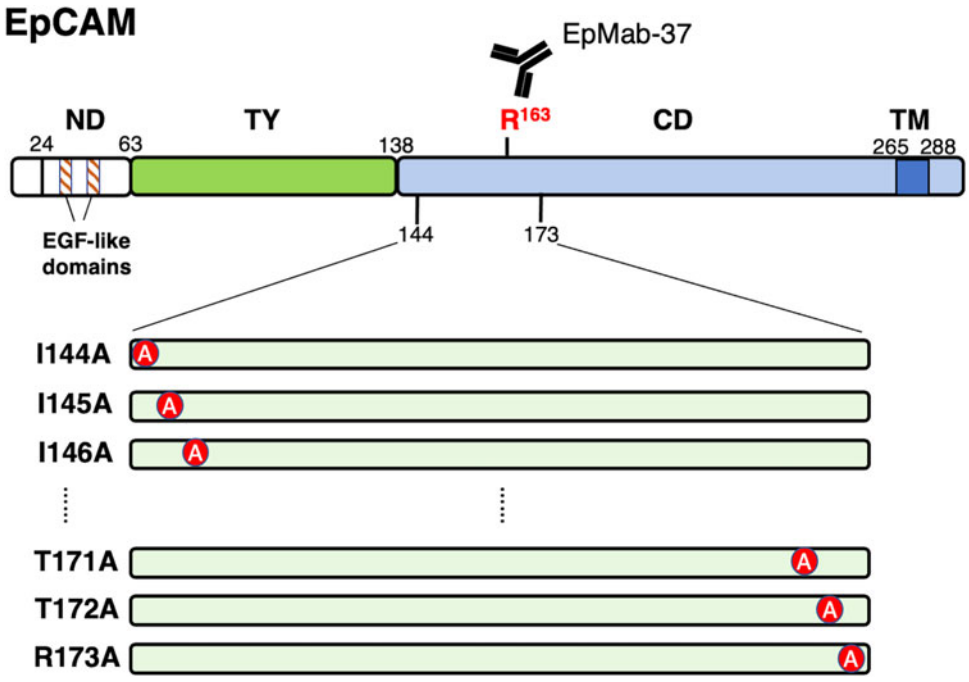

FIG. 1. Schematic illustration of EpCAM structure and the 1×alanine (glycine)-substituted EpCAM mutants. The N-terminal PA-tagged 1×alanine-substituted EpCAM mutants were transfected with CHO-K1 cells, and the stable transfectants were generated. The EpMab-37 epitope involves Arg163 of EpCAM. EpCAM, epithelial cell adhesion molecule; CD, C-terminal domain; CHO-K1, Chinese hamster ovary-K1; ND, N-terminal domain; TM, transmembrane domain; TY, thyroglobulin-like domain.

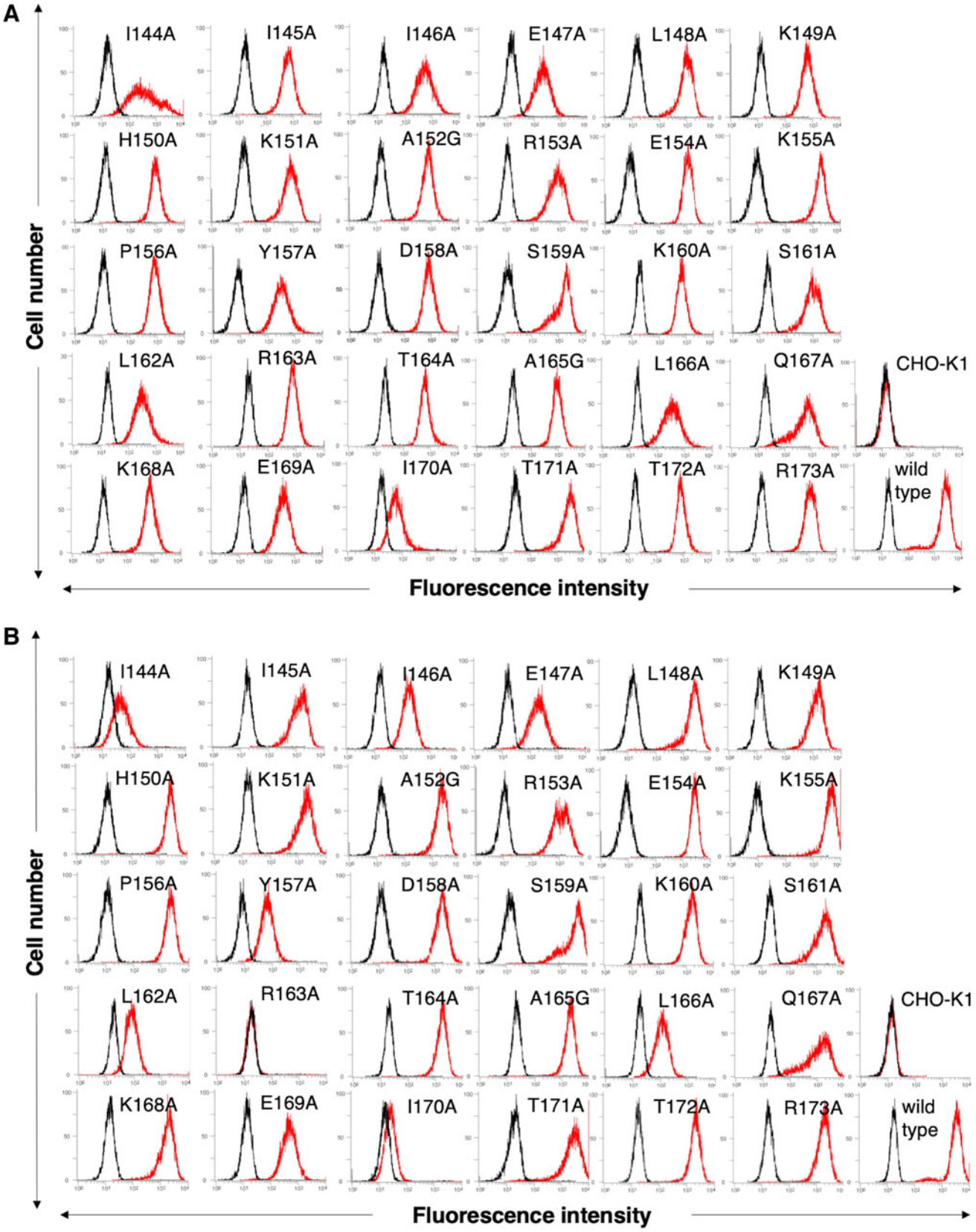

FIG. 2. Determination of the EpMab-37 epitope of EpCAM by flow cytometry using 1×Ala-scan method. **(A, B)** The wild type and 1×alanine-substituted EpCAM-overexpressed CHO-K1 were incubated with **(A)** NZ-1 (1 μg/mL) and **(B)** EpMab-37 (1 μg/mL), followed by treatment using Alexa Fluor 488-conjugated anti-rat and anti-mouse IgG, respectively; black line, negative control. 1×Ala-scan, 1×alanine scanning.

We first investigated the cell surface expression of the EpCAM mutants on CHO-K1 cells using NZ-1 (an anti-PA tag mAb), and confirmed the comparable expression levels (Fig. 2A). We next examined the reactivity of EpMab-37 to those EpCAM mutants, which were expressed in CHO-K1 cells. As shown in Figure 2B, EpMab-37 exhibited reaction with I144A, I145A, I146A, E147A, L148A, K149A, H150A, K151A, A152G, R153A, E154A, K155A, P156A, Y157A, D158A, S159A, K160A, S161A, L162A, T164A, A165G, L166A, Q167A, K168A, E169A, I170A, T171A, T172A, R173A, and wild type. In contrast, EpMab-37 did not react with EpCAM R163A.

Although the EpMab-37 reactivity completely disappeared in EpCAM R163A, several EpCAM mutants including I144A, I146A, Y157A, L162A, and I170A exhibited the reduced reactivity to EpMab-37. To confirm the involvement of these residues, we further established EpCAM mutants, in which sequential two aa were substituted into two alanine (or glycine) residues; this method is named as 2×Ala-scan method (Fig. 3).

We investigated the cell surface expression of the EpCAM mutants on CHO-K1 cells using NZ-1, and confirmed the comparable expression levels (Fig. 4A). We then examined the reactivity of EpMab-37 against those EpCAM mutants, which were expressed in CHO-K1 cells. As shown in Figure 4B, EpMab-37 exhibited reaction with I144A–I145A, I146A–E147A, L148A–K149A, H150A–K151A, A152G–R153A, E154A–K155A, P156A–Y157A, D158A–S159A, K160A–S161A, T164A–A165G, L166A–Q167A, K168A–E169A, I170A–T171A, T172A–R173A, and wild type. In contrast, EpMab-37 did not react with L162A–R163A. These results also indicated that Arg163 is the critical epitope of EpMab-37.

Discussion

We previously performed the 1×and 2×Ala-scan epitope mapping methods for an anti-CXCR6 mAb, clone Cx_6Mab-1,[26] using enzyme-linked immunosorbent assay (ELISA).[27] Since Cx_6Mab-1 can recognize linear peptide epitope, ELISA could be applied for the epitope mapping. In contrast, EpMab-37 never reacts with linear epitope,[19] suggesting that EpMab-37 recognizes the conformational epitope.

Therefore, we first performed 1×Ala-scan using one alanine (glycine)-substituted EpCAM mutants expressed on CHO-K1 cells (Fig. 1). We found that the EpMab-37 reactivity disappeared completely in EpCAM R163A (Fig. 2), suggesting that Arg163 is the most important residue of the EpMab-37 epitope. We further performed the epitope mapping of EpMab-37 using 2×Ala-scan, and confirmed that Arg163 is the only critical epitope of EpMab-37 (Fig. 4B).

The Arg163 is located on the α-helix of EpCAM CD, which is close to $_{167}$–QKEIT–$_{171}$ sequence recognized by MT201 (adecatumumab).[18] Alanine substitution in the sequence did not lose the EpMab-37 recognition (Fig. 2B), suggesting that EpMab-37 possesses a unique epitope and a different mode of actions. Recently, CTC expansion methods including two-dimensional long-term expansion, 3D organoids/spheroids culture, and xenografts have been developed to evaluate the character of CTCs.[28] Because EpCAM is an important antigen to collect CTCs, it would be worthwhile to investigate the effect of anti-EpCAM mAbs including EpMab-37 on the CTC expansion *in vitro* and metastasis *in vivo*. Furthermore, the relationship between the epitope and EpCAM-internalizing activity by anti-EpCAM mAbs should be evaluated for the development of antibody–drug conjugates.

Anti-EpCAM mAb can be applied to a bispecific antibody with anti-MET mAb.[29] Because MET is one of the important oncogenes that promote tumorigenesis and metastasis,[30] MET-targeting antibody therapeutics have been desired for clinic.[31] However, the clinical development of MET-targeted antibodies has been challenging because bivalent antibodies exhibit agonistic properties, whereas monovalent antibodies lack potency and the capacity to downregulate MET.[32,33]

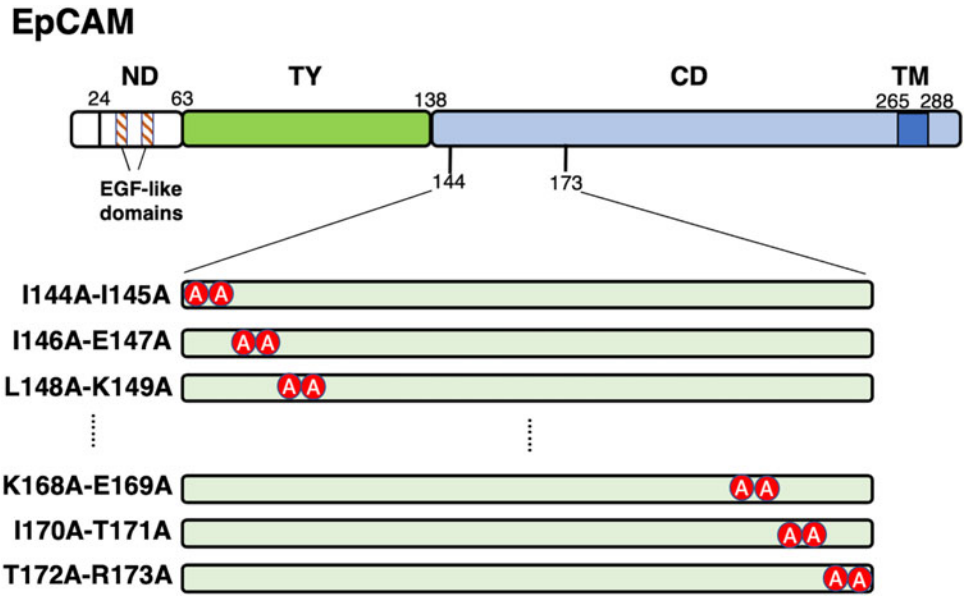

FIG. 3. Schematic illustration of the 2×alanine-substituted EpCAM mutants. The N-terminal PA-tagged 2×alanine-substituted EpCAM mutants were transfected with CHO-K1 cells, and the stable transfectants were generated.

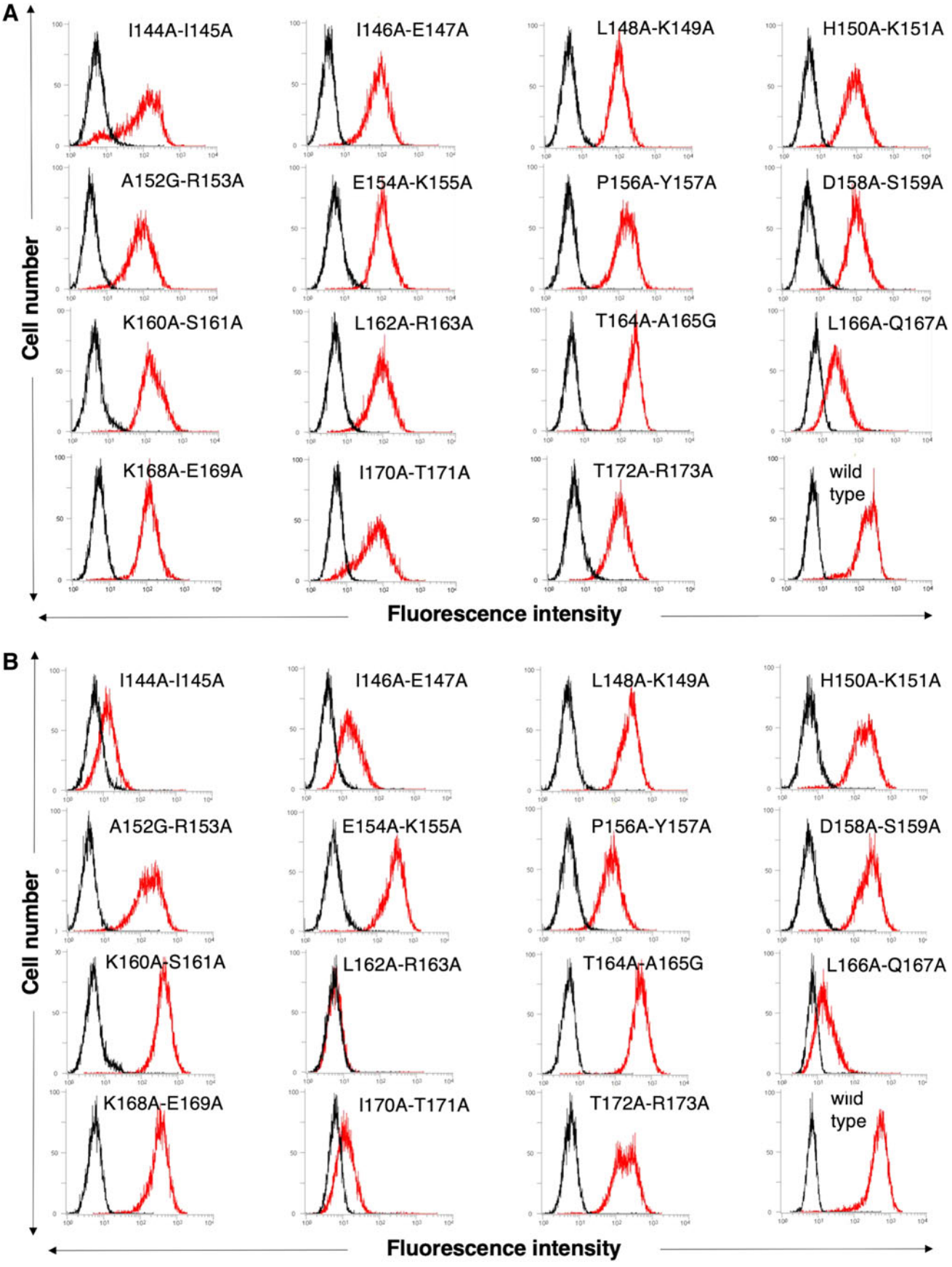

FIG. 4. Determination of the EpMab-37 epitope of EpCAM by flow cytometry using 2×Ala-scan method. **(A, B)** The wild type and 2×alanine-substituted EpCAM-overexpressed CHO-K1 cells were incubated with **(A)** NZ-1 (1 μg/mL) and **(B)** EpMab-37 (1 μg/mL), followed by treatment using Alexa Fluor 488-conjugated anti-rat and anti-mouse IgG, respectively; black line, negative control. 2×Ala-scan, 2×alanine scanning.

MM-131 is a bispecific antibody that is monovalent for MET, but exhibits high avidity by concurrently binding to EpCAM using single chain Fv of MOC31.[34] MM-131 exhibits antagonistic antibody that blocks ligand-dependent and ligand-independent MET signaling by inhibiting HGF binding to MET and inducing receptor downregulation.[29] In the future study, we would like to apply EpMab-37 for the combination therapy with MET or bispecific mAbs.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Yukinari Kato
Department of Molecular Pharmacology
Tohoku University Graduate School of Medicine
2-1, Seiryo-machi, Aoba-ku
Sendai 980-8575
Japan

E-mail: yukinari.kato.e6@tohoku.ac.jp

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