MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 40, Number 4, 2021

DOI: 10.1089/mab.2021.0014

Short Communications

# Epitope Mapping of an Antihuman EGFR Monoclonal Antibody (EMab-134) Using the REMAP Method

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The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that plays an important role in normal epidermal cell physiology. EGFR is overexpressed in cancer cells and has a number of mutations that implicate tumor malignancy, development, and poor patient prognosis; thus, EGFR is an attractive target for cancer therapy. At present, anti-EGFR monoclonal antibodies (mAbs) have been approved and are used for treating patients with a variety of EGFR-expressing cancers. Epitope mapping is important in identifying the therapeutic mechanism of anti-EGFR mAbs; however, the development of epitope mapping techniques lags behind the development of antimolecular target mAbs, including anti-EGFR mAbs. Hence, in this study, a novel epitope mapping method, RIEDL insertion for epitope mapping (REMAP) method, was developed. The results of this study demonstrated that the critical epitope of anti-EGFR mAb EMab-134 is Gly378, Asp379, Ser380, Phe381, Thr382, His383, Thr384, Pro385, and Pro386 of EGFR. The REMAP method could be useful for determining the critical epitope of functional mAbs against many target molecules.

**Keywords:** EGFR, EMab-134, epitope mapping, monoclonal antibody, RIEDL tag



## Introduction

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor, closely related to human epidermal receptor 2 (HER2), HER3, and HER4.[1,2] EGFR is activated by ligand binding and it promotes cell progression by activating the ERK, MAPK, AKT-PI3K, and PLC-γ1-PKC pathways.[3–6] EGFR is overexpressed in various cancers and is usually associated with the survival, proliferation, invasion, and drug resistance of cancer cells.[7,8] Therefore, the EGFR expression is a poor prognostic factor for cancer patients and EGFR is an attractive target for cancer therapy. Although EGFR-targeting therapies, such as EGFR tyrosine kinase inhibitors and anti-EGFR monoclonal antibodies (mAbs), show some antitumor efficacy in clinical trials, tumors can evolve with EGFR-resistant mutations, thereby causing failure of these therapies.[9–14] Thus, EGFR-targeting drugs are needed to overcome these mutations.

Four anti-EGFR antibodies, cetuximab, panitumumab, nimotuzumab, and necitumumab, have been developed for clinical use in the treatment of colorectal cancer, head and neck squamous cell carcinoma, glioblastoma, gastric cancer, and squamous nonsmall cell lung cancer.[15–22] Epitope mapping of these mAbs using structural analysis reveals that they bind to different amino acid (aa) residues of domain III of EGFR and inhibit binding of the EGFR ligands, such as EGF.[23–26] The structural analysis revealed that panitumumab binds to the cetuximab-resistant EGFR K467T mutant, whereas necitumumab binds to cetuximab- and panitumumab-resistant EGFR mutants.[23,24,26] Therefore, epitope mapping is important in determining the therapeutic and molecular mechanisms of mAbs.

Epitopes of mAbs are generally divided into two types: linear and conformational epitopes. Linear epitopes are formed by continuous aa residues (≤16 aa), whereas conformational epitopes are not continuous and are formed by three-dimensional peptides combined by protein folding.[27] There are several conformational epitope mapping methods, such as X-ray cocrystallography, cryogenic electron microscopy, site-directed mutagenesis mapping, high-throughput shotgun mutagenesis epitope mapping, and hydrogen–deuterium exchange mass spectrometry.[28–32] X-ray cocrystallography and cryogenic electron microscopy can precisely determine the antibody binding sites, but these methods are highly technical, time consuming, and expensive. In site-directed mutagenesis mapping and high-throughput shotgun mutagenesis epitope mapping, it is challenging to map a part of a conformational epitope because a single aa residue mutation is not enough to disrupt the antibody–antigen interaction. Currently, large number of mAbs have been obtained as

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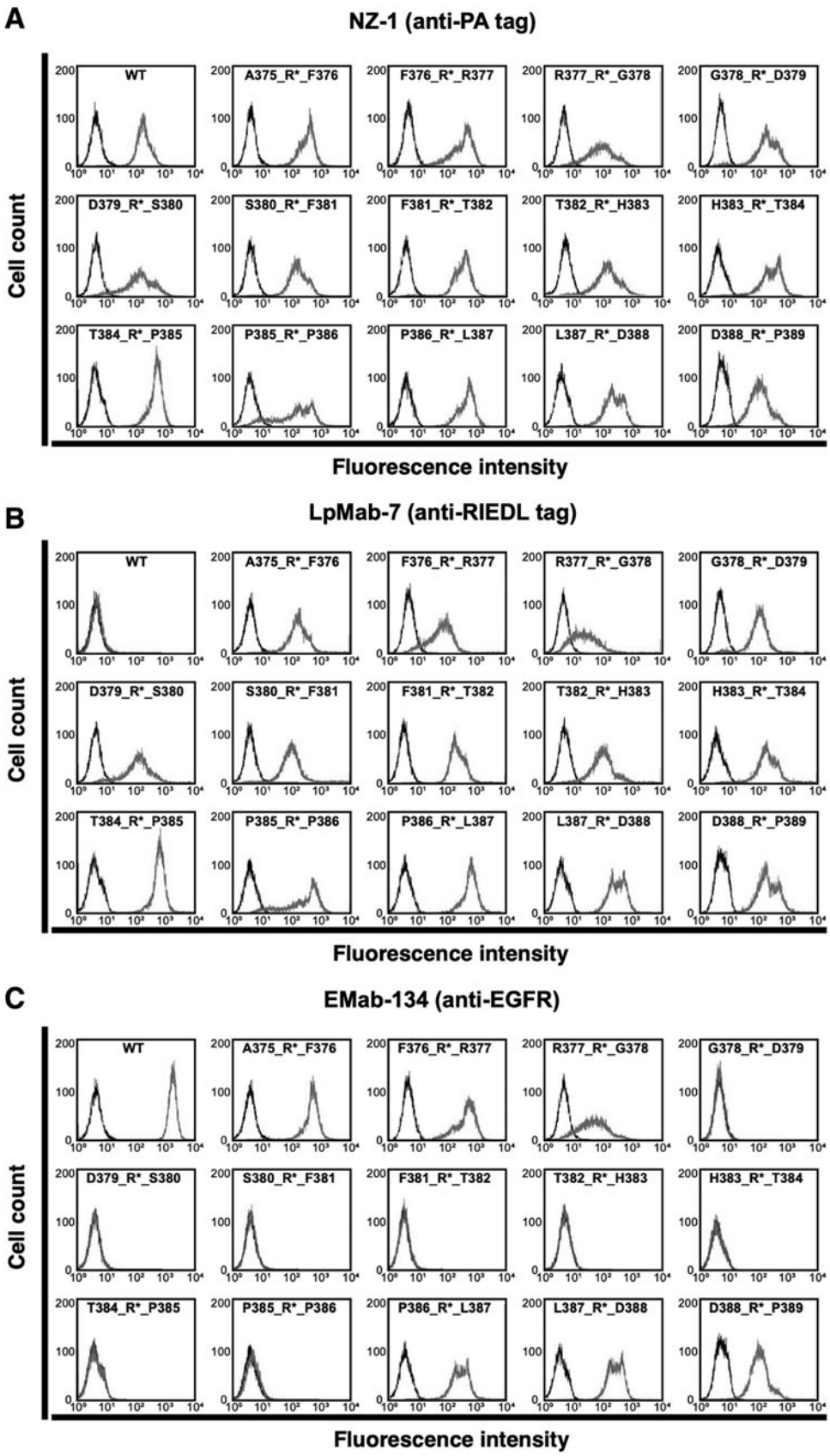


**FIG. 1.** Epitope mapping using RIEDL tag-inserted mutants of EGFR/dN313. The RIEDL tag-inserted mutants were incubated with **(A)** NZ-1 (an anti-PAtag mAb), **(B)** LpMab-7 (an anti-RIEDL tag mAb), or **(C)** EMab-134. Black lines: control (without 1st mAb). EGFR, epidermal growth factor receptor; mAb, monoclonal antibody.

therapeutic drugs against many diseases; however, the speed of epitope mapping techniques has not kept pace with the increasing number of isolated mAbs.[31,33]

In this study, a novel epitope mapping technique using the RIEDL tag system[34] was developed. Then, the epitope of anti-EGFR mAb (clone EMab-134)[35] was characterized using the RIEDL insertion for epitope mapping (REMAP) method.

## Materials and Methods

### *Cell lines*

Chinese hamster ovary (CHO)-K1 cells were obtained from the America Type Culture Collection (ATCC, Manassas, VA). The EGFR mutation plasmids were transfected into the CHO-K1 cells using the Neon Transfection System (Thermo Fisher Scientific, Inc., Waltham, MA) and stable transfectants were sorted using a cell sorter (SH800; Sony Corp., Tokyo, Japan). The CHO-K1 cells and transfectants were cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, and 0.25 $\mu$g/mL amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere containing 5% $CO_2$. The transfectants were cultivated in a medium containing 0.5 mg/mL Zeocin (InvivoGen, San Diego, CA).

### *Plasmid preparation*

The EGFR open reading frames were amplified as described previously.[36] The EGFR deletion mutants (EGFR/dN313) were produced using the HotStar HiFidelity Polymerase Kit (Qiagen, Inc., Hilden, Germany) with oligonucleotides containing the desired mutations. The PA tag (GVAMPGAEDDVV) was added at the N-terminus,[37] which is recognized by an anti-PA tag mAb (NZ-1).[38] The RIEDL tag[34] was inserted into the EGFR sequence using the HotStar HiFidelity Polymerase Kit with oligonucleotides containing the RIEDL tag insertions at the desired position in $_{375}$-AFRGDSFTHTPPLDP-$_{389}$ of EGFR/dN313. For example, Ala375_RIEDL_Phe376 (A375_R*_F376) was produced by inserting the RIEDL sequence between Ala375 and Phe376 of EGFR/dN313. The polymerase chain reaction fragments with the desired mutations were inserted into the pCAG-Ble vector using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan). The following RIEDL tag insertion mutants were produced: Ala375_RIEDL_Phe376 (A375_R*_F376), Phe376_RIEDL_Arg377 (F376_R*_R377), Arg377_RIEDL_Gly378 (R377_R*_G378), Gly378_RIEDL_Asp379 (G378_R*_D379), Asp379_RIEDL_Ser380 (D379_R*_S380), Ser380_RIEDL_Phe381 (S380_R*_F381), Phe381_RIEDL_Thr382 (F381_R*_T382), Thr382_RIEDL_His383 (T382_R*_H383), His383_RIEDL_Thr384 (H383_R*_T384), Thr384_RIEDL_Pro385 (T384_R*_P385), Pro385_RIEDL_Pro386 (P385_R*_P386), Pro386_RIEDL_Leu 387 (P386_R*_L387), Leu387_RIEDL_Asp388 (L387_R*_D388), and Asp388_RIEDL_Pro389 (D388_R*_P389).

### *Flow cytometry*

The cells were harvested by a brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing the cells with 0.1% bovine serum albumin in phosphate-buffered saline, they were treated with primary mAbs (1 or 10 $\mu$g/mL) for 30 minutes at 4°C and then with Alexa Fluor 488-conjugated antimouse immunoglobulin G (IgG) or antirat IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp.).

## Results and Discussion

In our previous study, the critical epitope of an anti-EGFR mAb (EMab-134)[35] was determined to be $_{377}$-RGDSFTHTPP-$_{386}$ from domain III of the extracellular region of EGFR using site-directed mutagenesis epitope mapping.[39] In this study, the epitope of EMab-134 was characterized using a novel epitope mapping technique: REMAP method.

We produced 14 EGFR/dN313 transfectants (an N-terminal PA tag and EGFR from aa 313 to 1210), in which a RIEDL tag was inserted into the expected epitope region at each possible position of $_{375}$-AFRGDSFTHTPPLDP-$_{389}$ (Fig. 1). The LpMab-7 (an anti-RIEDL tag mAb) recognized the five aa-long RIEDL tag.[34] For example, we produced Ala375_RIEDL_Phe376 (A375_R*_F376) by inserting the RIEDL sequence between Ala375 and Phe376 of EGFR/dN313.

Flow cytometry analysis showed that positive control NZ-1 (an anti-PA tag mAb) detected wild type (WT) and 14 mutants of EGFR/dN313 (Fig. 1A). Because RIEDL sequence was not inserted into WT, LpMab-7 did not react with WT, but reacted with 14 mutants of EGFR/dN313 (Fig. 1B). In contrast, EMab-134 did not react with eight mutants, such as Gly378_RIEDL_Asp379 (G378_R*_D379), Asp379_RIEDL_Ser380 (D379_R*_S380), Ser380_RIEDL_Phe381 (S380_R*_F381), Phe381_RIEDL_Thr382 (F381_R*_T382), Thr382_RIEDL_His383 (T382_R*_H383), His383_RIEDL_Thr384 (H383_R*_T384), Thr384_RIEDL_Pro385 (T384_R*_P385), and Pro385_RIEDL_Pro386 (P385_R*_P386), although it strongly detected six mutants, such as Ala375_RIEDL_Phe376 (A375_R*_F376), Phe376_RIEDL_Arg377 (F376_R*_R377), Arg377_RIEDL_Gly378

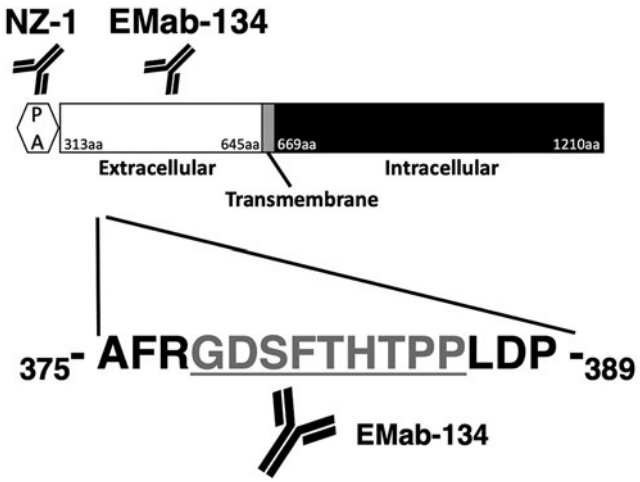


**FIG. 2.** Schematic illustration of EMab-134 epitopes. ''GDSFTHTPP'' is a critical epitope of EMab-134. PA, PA tag.

(R377_R*_G378), Pro386_RIEDL_Leu387 (P386_R*_L387), Leu387_RIEDL_Asp388 (L387_R*_D388), and Asp388_RIEDL_Pro389 (D388_R*_P389) (Fig. 1C), indicating that EMab-134 might bind to EGFR through nine aas ($_{378}$-GDSFTHTPP-$_{386}$). These results are summarized in Figure 2.

As described in this study, the REMAP method may also be suitable for the mapping of linear and conformational epitopes of a number of anti-EGFR mAbs, including those used in preclinical or clinical trials. This could reveal novel therapeutic mechanisms of anti-EGFR mAbs. Furthermore, there are a number of mAbs for which the antigen binding site has not been identified. The REMAP method might be able to characterize the epitope of these mAbs in a future study.

### 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 Grant Nos. JP21am0401013 (Y.K.) and JP21am0101078 (Y.K.) and by Japan Society for the Promotion of Science (JSPS) KAKENHI under Grant Nos. 21K16322 (M.S.), 21K07168 (M.K.K.), 21K15523 (T.A.), and 21K07705 (Y.K.).



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*Received: March 25, 2021*
*Accepted: May 17, 2021*