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# Antitumor Activities in Mouse Xenograft Models of Canine Mammary Gland Tumor by Defucosylated Mouse-Dog Chimeric Anti-Epidermal Growth Factor Receptor Antibody (E134Bf)

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The epidermal growth factor receptor (EGFR) contributes to tumor malignancy through gene amplification and/or protein overexpression. In our previous study, we developed an anti-human EGFR (hEGFR) monoclonal antibody (mAb), clone EMab-134 (mouse IgG<sub>1</sub>, kappa), which specifically detects both hEGFR and dog EGFR (dEGFR). The defucosylated mouse IgG<sub>2a</sub> version of EMab-134 exhibits antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) in dEGFR-overexpressed CHO-K1 (CHO/dEGFR) cells and antitumor activities in mouse xenografts of CHO/dEGFR cells. In this study, we produced a defucosylated mouse-dog chimeric anti-EGFR mAb (E134Bf), and the reactivity of E134Bf against a canine mammary gland tumor cell line (SNP) was examined by flow cytometry. Furthermore, E134Bf highly exerted ADCC and CDC for SNP cells. The administration of E134Bf with canine mononuclear cells significantly suppressed the SNP xenograft growth. These results suggest that E134Bf exerts antitumor effects against dEGFR-expressing canine mammary gland tumors and could be valuable as part of an antibody treatment regimen for them.

**Keywords:** EGFR, mouse-dog chimeric antibody, ADCC, CDC, antitumor activity

## Introduction

CANINE MAMMARY GLAND tumor is the most common neoplasm in female dogs. It has several similarities with human breast cancer, including histological and molecular subtypes, metastasis, and response to treatment.<sup>(1)</sup> Similar to human breast cancer, canine mammary gland tumors can be classified into luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-overexpressing, basal-like, or normal-like according to the immunohistochemistry results. Luminal A (estrogen receptor [ER]-positive and HER2-negative) was the most frequent subtype.<sup>(2)</sup> ER-positive

canine mammary tumors have been reported as complex carcinomas that include both luminal and myoepithelial cells. In contrast, ER-tumors are classified into simple carcinomas arise from genomic aberrations.<sup>(3)</sup> Therefore, it is suggested as a model for studying human breast cancer.

Epidermal growth factor receptor (EGFR) is a type I glycoprotein that plays crucial roles in promotion of cell proliferation and survival through activation of RAS-RAF-MEK-ERK and PI3K/Akt pathways.<sup>(4)</sup> Overexpression and mutations of EGFR have been identified with various cancers in both human and dog.<sup>(5-9)</sup> EGFR expression is associated with poor prognosis in canine mammary cancer.<sup>(10)</sup> Since

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human EGFR (hEGFR) and dog EGFR (dEGFR) have 91% amino acid homology,<sup>(11)</sup> the therapeutic methods targeting hEGFR is thought to be available for canine tumor treatment.

In our previous study, we developed an anti-hEGFR monoclonal antibody (mAb), clone EMab-134 (mouse IgG<sub>1</sub>, kappa).<sup>(12)</sup> EMab-134 can recognize both hEGFR and dEGFR.<sup>(13)</sup> The 134-mG<sub>2a</sub> mouse IgG<sub>2a</sub> type of EMab-134, exerted antitumor activities against human oral squamous cell carcinoma.<sup>(14)</sup> Furthermore, defucosylated type of 134-mG<sub>2a</sub> (134-mG<sub>2a</sub>-f) possesses antitumor effects against dEGFR-overexpressing cells.<sup>(13)</sup>

In this study, we investigated whether a defucosylated mouse-dog chimeric anti-EGFR mAb (E134Bf) possesses antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antitumor activities using a canine mammary gland tumor model.

## Materials and Methods

### Cell lines

A canine mammary gland tumor cell line, SNP, was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University (Miyagi, Japan).<sup>(15)</sup> SNP was cultured in RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA), 100 units/mL of penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Nacalai Tesque, Inc.). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### Antibodies

Anti-hEGFR mAb EMab-134 was developed as previously described.<sup>(12)</sup> To generate E134B, we subcloned V<sub>H</sub> complementary DNA (cDNA) of EMab-134 and C<sub>H</sub> of dog IgGB into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), along with V<sub>L</sub> cDNA of EMab-134 and C<sub>L</sub> cDNA of dog kappa light chain into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation), respectively. The vector of E134B was transfected into BINDS-09 cells (FUT8-deficient ExpiCHO-S cells) using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.). The resulting mAb, E134Bf, was purified with Protein G-Sepharose (GE Healthcare Biosciences, Pittsburgh, PA). Dog IgG was purchased from Jackson ImmunoResearch, Inc. (West Grove, PA).

### Animals

All animal experiments were performed following relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments for the antitumor activity were approved by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (permit No. 2021-021). Mice were maintained in a specific pathogen-free environment (23°C ± 2°C, 55% ± 5% humidity) on an 11 hours light/13 hours dark cycle with food and water supplied *ad libitum* across the experimental period. Mice were monitored for health and weight every 2–5 days during the 3-week period of each experiment. We determined the loss of original body weight to a point >25% and/or a max-

imum tumor size >3000 mm<sup>3</sup> as humane endpoints for euthanasia. Mice were euthanized by cervical dislocation; death was verified by respiratory and cardiac arrest.

### Flow cytometry

SNP cells were harvested by brief exposure to 0.25% trypsin/1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai Tesque, Inc.). After washing with blocking buffer of 0.1% bovine serum albumin in phosphate-buffered saline (PBS), cells were treated with 1 µg/mL of E134Bf, or control blocking buffer for 30 minutes at 4°C. Then, cells were incubated in FITC-conjugated anti-dog IgG at a dilution of 1:1000 (Thermo Fisher Scientific, Inc.) for 30 minutes at 4°C. Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).

### Determination of binding affinity

SNP cells were suspended in 100 µL of serially diluted E134Bf (0.006–100 µg/mL) followed by FITC-conjugated anti-dog IgG (1:200). Fluorescence data were collected using the EC800 Cell Analyzer. The dissociation constant ( $K_D$ ) was calculated by fitting binding isotherms to built-in one-site binding models in GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA).

### ADCC

Canine mononuclear cells (MNC) were obtained from Yamaguchi University and resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS to be used as effector cells. Target SNP cells were labeled with 10 µg/mL Calcein AM (Thermo Fisher Scientific, Inc.) and resuspended in the same medium. The target cells (2 × 10<sup>4</sup> cells/well) were plated in 96-well plates and mixed with effector cells (effector/target cells ratio, 50), 100 µg/mL of E134Bf, or control dog IgG. Following 4.5-hour incubation at 37°C, the release of Calcein into the supernatant was measured in each well. The fluorescence intensity was determined using a microplate reader (Power Scan HT; BioTek Instruments, Inc., Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Cytolytic activity (% lysis) was calculated as follows: % lysis = (E – S)/(M – S) × 100, where “E” is the fluorescence measured in combined cultures of target and effector cells, “S” is the spontaneous fluorescence of target cells only, and “M” is the maximum fluorescence measured following the lysis of all cells with a buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4), and 10 mM of EDTA.

### CDC

SNP cells were labeled with 10 µg/mL Calcein AM and resuspended in the medium. They were then plated in 96-well plates at 2 × 10<sup>4</sup> cells/well with rabbit complement (final dilution 1:10; Low-Tox-M Rabbit Complement; Cedarlane Laboratories, Hornby, Canada) and 100 µg/mL of E134Bf or control dog IgG. Following 4 hours of incubation at 37°C, we measured Calcein release into the supernatant for each well. Fluorescence intensity was calculated as described in the ADCC section above.

### Antitumor activity of E134Bf in xenografts of SNP cells

A total of 16 female BALB/c nude mice (5 weeks old, weighing 14–17 g) were purchased from Charles River Laboratories, Inc. (Kanagawa, Japan), and used in experiments once they reached 7 weeks of age. SNP cells ( $0.3 \text{ mL}$  of  $1.33 \times 10^8$  cells/mL in DMEM) were mixed with  $0.5 \text{ mL}$  BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, San Jose, CA);  $100 \mu\text{L}$  of this suspension ( $5 \times 10^6$  cells) was injected subcutaneously into the left flanks of the mice.

On day 6 postinoculation,  $100 \mu\text{g}$  of E134Bf ( $n=8$ ) or control dog IgG ( $n=8$ ) in  $100 \mu\text{L}$  PBS were injected intraperitoneally. Additional antibody inoculations were performed on days 15 and 23. Canine MNC ( $6.3 \times 10^5$  cells), which were obtained from Yamaguchi University, were injected surrounding the tumors treated with both dog IgG and E134Bf on days 6, 15, and 23. At 28 days following cell implantation, all mice were euthanized by cervical dislocation. Tumor diameters and volumes were determined as previously described.<sup>(13)</sup>

### Statistical analyses

All data are expressed as mean  $\pm$  standard error of the mean. Statistical analysis was conducted with Tukey's test for ADCC and CDC, and Welch's *t* test for tumor weight. Analysis of variance and Sidak's multiple comparisons tests were conducted for tumor volume and mouse weight. All calculations were performed using GraphPad Prism 8 (GraphPad Software, Inc.). A *p*-value of  $<0.05$  was considered statistically significant.

## Results

### Flow cytometry analysis against a canine mammary gland tumor cell line, SNP cells using E134Bf

In our previous study, an anti-hEGFR mAb, E134, recognized dEGFR-overexpressed CHO/dEGFR cells, indicating that E134 crossreacts with dEGFR.<sup>(13)</sup> In this study, we produced the defucosylated mouse-dog chimeric mAb from E134 (E134Bf) and demonstrated that E134Bf detected SNP cells (Fig. 1A), indicating that E134Bf detect endogenous dEGFR expressed on SNP cells.

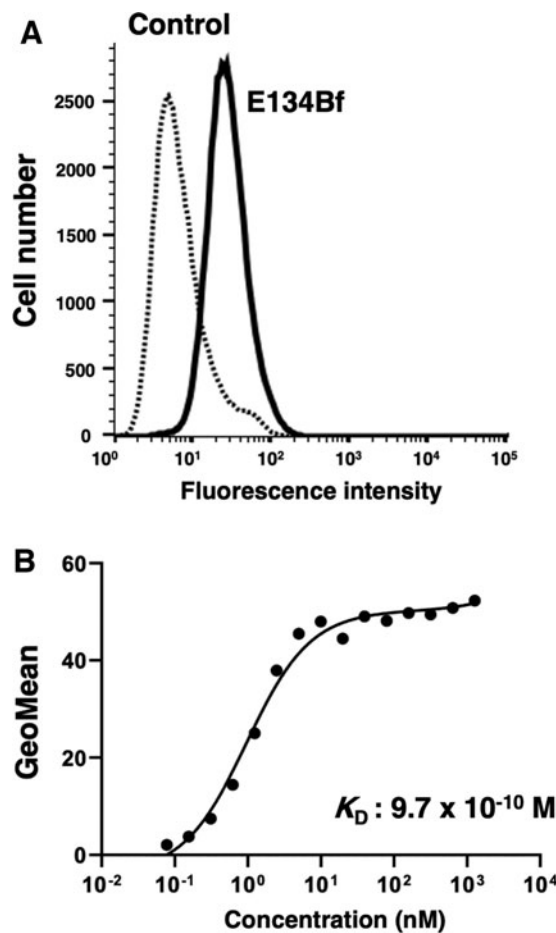
### Determination of the binding affinity

A kinetic analysis of the interactions of E134Bf with SNP cells was performed via flow cytometry. As shown in Figure 1B, the  $K_D$  for the interaction of E134Bf with SNP cells was  $9.7 \times 10^{-10} \text{ M}$ , suggesting that E134Bf shows high affinity for SNP cells.

### E134Bf-mediated ADCC and CDC in SNP cells

We next investigated whether E134Bf has ADCC against SNP cells using canine MNC as effector. As shown in Figure 2A, E134Bf showed ADCC (37.0% cytotoxicity) against SNP cells more potent than dog IgG (14.4% cytotoxicity) and PBS (10.2% cytotoxicity).

We then investigated whether E134Bf has CDC against SNP cells. As shown in Figure 2B, E134Bf elicited a higher degree of CDC (37.7% cytotoxicity) in SNP cells compared with that elicited by dog IgG (14.4% cytotoxicity) and PBS (9.4% cytotoxicity). These results demonstrated that E134Bf exerts ADCC and CDC against dEGFR-expressing SNP cells.

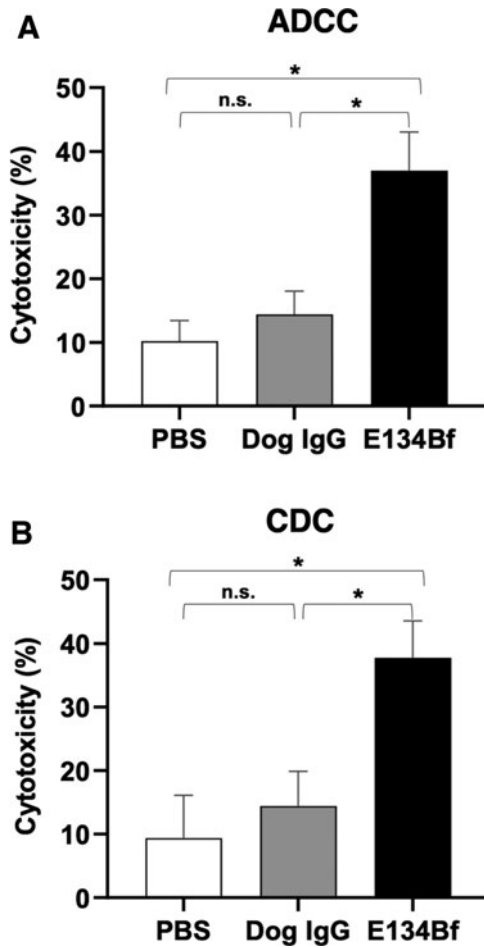


**FIG. 1.** Flow cytometry using E134Bf. (A) SNP cells were treated with E134Bf or buffer control, followed by FITC-conjugated anti-dog IgG. (B) Determination of the binding affinity of E134Bf for SNP cells using flow cytometry. SNP cells were suspended in  $100 \mu\text{L}$  of serially diluted E134Bf, followed by the addition of FITC-conjugated anti-dog IgG. Fluorescence data were collected using the EC800 Cell Analyzer.

### Antitumor activities of E134Bf in the mouse xenografts of SNP cells

In the SNP xenograft models, E134Bf ( $100 \mu\text{g}$ ) and control dog IgG ( $100 \mu\text{g}$ ) were injected intraperitoneally into mice on days 6, 15, and 23 following the injection of SNP cells. Canine MNC were also injected surrounding the tumors in both groups on days 6, 15, and 23. The tumor volume was measured on days 6, 12, 15, 17, 23, 26, and 28 after the injection. The administration of E134Bf resulted in a significant reduction in tumor development on day 12 ( $p < 0.05$ ), day 15 ( $p < 0.01$ ), day 17 ( $p < 0.01$ ), day 23 ( $p < 0.01$ ), day 26 ( $p < 0.01$ ), and day 28 ( $p < 0.01$ ) compared with that of the control dog IgG (Fig. 3).

The administration of E134Bf resulted in a 35% reduction of tumor volume compared with that of the control dog IgG on day 28 postinjection. Furthermore, the tumor weight of the E134Bf-treated mice was significantly lower than that of control dog IgG-treated mice (28% reduction;  $p < 0.05$ , Fig. 4A). The resected tumors are shown in Figure 4B. Total



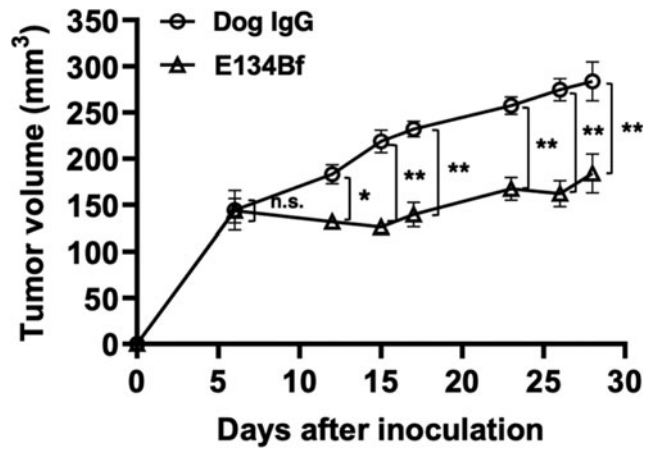
**FIG. 2.** Evaluation of ADCC and CDC elicited by E134Bf. **(A)** ADCC elicited by E134Bf, dog IgG, or PBS targeting SNP cells. Asterisks indicate statistical significance ( $*p < 0.05$ , n.s.; Tukey's test). **(B)** CDC elicited by E134Bf, dog IgG, or PBS targeting SNP cells. Values are means  $\pm$  SEM. Asterisks indicate statistical significance ( $*p < 0.05$ , n.s.; Tukey's test). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; n.s., not significant; PBS, phosphate-buffered saline; SEM, standard error of the mean.

body weights of the two groups did not change significantly (Fig. 5A). The mice on day 28 are shown in Figure 5B.

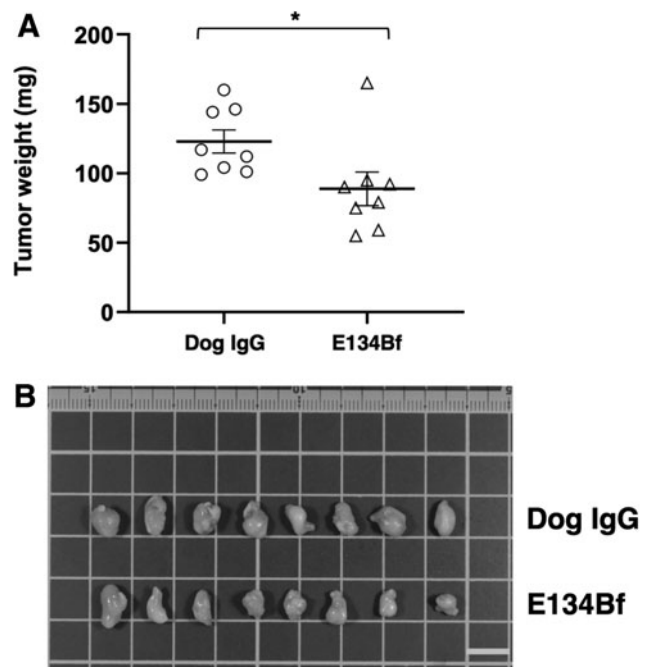
Taken together, these results indicate that the administration of E134Bf effectively suppresses the tumor growth of SNP xenografts.

## Discussion

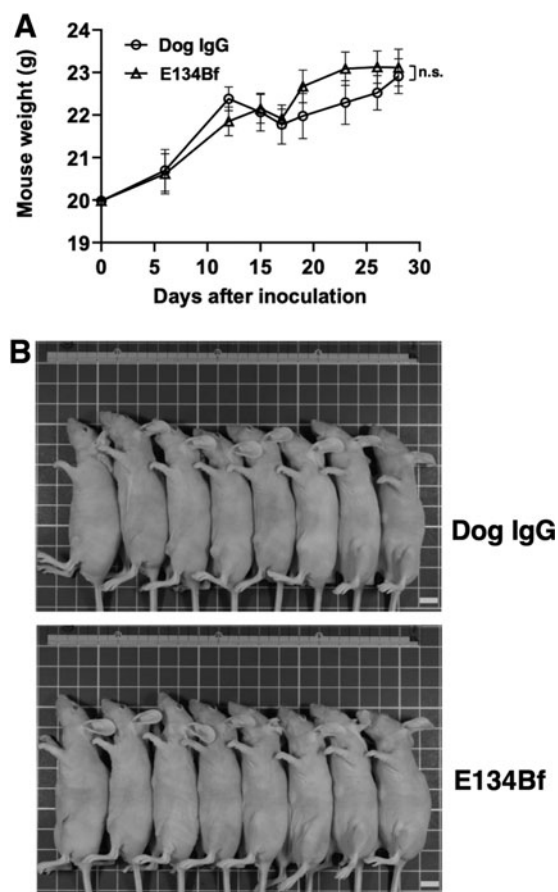
This study demonstrated antitumor activities of a defucosylated mouse-dog chimeric mAb of anti-hEGFR mAb (E134Bf) on *in vitro* and *in vivo* in canine mammary gland tumor cells, which express endogenous dEGFR. EGFR is one of the important oncoproteins that promotes tumor cell proliferation and survival.<sup>(4)</sup> Therapeutic agents targeting EGFR, including kinase inhibitors and antibody drugs, have been developed and clinically applied to human.<sup>(16,17)</sup> The anti-EGFR antibody drugs, including cetuximab and panitumumab, have similar binding affinities to EGFR, but have



**FIG. 3.** Evaluation of tumor volume in SNP xenografts. SNP cells ( $5 \times 10^6$  cells) were injected subcutaneously into the left flank. On day 6, 100  $\mu$ g of E134Bf or dog IgG in 100  $\mu$ L PBS were injected intraperitoneally into mice; additional antibodies were then injected on days 15 and 23. Canine mononuclear cells ( $6.3 \times 10^5$  cells), which were obtained from Yamaguchi University, were injected surrounding the tumors treated with both dog IgG and E134Bf on days 6, 15, and 23. The tumor volume was measured on days 6, 12, 15, 17, 23, 26, and 28 after the tumor inoculation. Values are means  $\pm$  SEM. Asterisks indicate statistical significance ( $**p < 0.01$ ,  $*p < 0.05$ , n.s.; ANOVA and Sidak's multiple comparisons test). ANOVA, analysis of variance.



**FIG. 4.** Evaluation of tumor weight in SNP xenografts. **(A)** SNP xenografts were resected from E134Bf and dog IgG groups. Tumor weight on day 28 was measured. Values are means  $\pm$  SEM. Asterisk indicates statistical significance ( $*p < 0.05$ , Welch's *t* test). **(B)** The appearance of resected tumors from E134Bf and dog IgG groups. Scale bar, 1 cm.



**FIG. 5.** Evaluation of body weights in SNP xenografts. **(A)** Body weights of mice implanted with SNP xenografts were recorded on days 6, 12, 15, 17, 19, 23, 26, and 28 after the tumor inoculation. (n.s.). **(B)** The appearance of mice from E134Bf and dog IgG groups. Scale bar, 1 cm.

different epitopes on the EGFR.<sup>(18–20)</sup> These mAbs possess higher affinity to EGFR than EGF and have neutralization ability to inhibit the EGFR signaling pathways.<sup>(21)</sup>

Basal type breast carcinoma includes triple negative breast carcinoma (TNBC) characterized by the absence or low expression of ER, progesterone receptor, and HER2.<sup>(22)</sup> TNBC has a poor prognosis compared to other breast cancer subtypes. Currently, molecular targeted drugs, including PD-L1/PD-1 inhibitors, PARP inhibitors, and anti-TROP2 antibody drug conjugates have received FDA approval.<sup>(23)</sup> Growing number of evidences suggest that EGFR pathway plays critical roles in the malignant progression of TNBC.<sup>(24,25)</sup> EGFR activation/amplification is detected in ~25%–50% of TNBC.<sup>(26,27)</sup> EGFR inhibition by antibody drug and/or EGFR tyrosine kinase inhibitors should be effective in the treatment of EGFR-driven TNBC.

Although EGFR is overexpressed in TNBC, clinical trials with EGFR inhibitors, including cetuximab, have been unsuccessful.<sup>(28)</sup> Novel approach to inhibit EGFR signaling is thought to be required. In this study, we could detect dEGFR expression in SNP cells (Fig. 1A). Furthermore, E134Bf exerts antitumor effects through ADCC and CDC activities (Figs. 2 and 3). Therefore, this model is useful to evaluate the EGFR targeting drugs and/or combination therapy with antitumor drugs.

Approved drugs for human are often used for canine cancer therapy due to their similarities.<sup>(29,30)</sup> E134Bf was originally developed using hEGFR as immunogen.<sup>(12)</sup> In case of antibody drug, the development of chimeric antibody is essential to evaluate the effect in dog. In this study, we developed mouse-dog chimeric antibody and produced defucosylated form to potentiate the ADCC activity. E134Bf possesses high affinity to dEGFR (Fig. 1B). Recently, we have determined an epitope of E134Bf between G378 to P386 of hEGFR<sup>(31)</sup> which is conserved in large part between human and dog. Since E134Bf exerted ADCC and antitumor effect in the presence of canine MNC (Figs. 2A and 3), E134Bf is expected to apply the clinical use. Further studies are required for the investigation of antitumor activity of E134Bf to spontaneously develop canine cancer and the establishment of technique to diagnose canine cancer, including dEGFR expression.

#### Author Disclosure Statement

The authors have no conflict of interest.

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