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*Article*

# Antitumor activity of an anti-EGFR/HER2 bispecific antibody in a mouse xenograft model of canine osteosarcoma

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**Abstract:** The overexpression of epidermal growth factor receptors (EGFRs) has been reported in various human tumors, including breast, gastric, lung, colorectal, and pancreatic cancers. Humanized anti-EGFR and human epidermal growth factor receptor 2 (HER2) monoclonal antibodies (mAbs) have been shown to improve patients' survival. Canine tumors resemble human tumors in the initiation and progression. We previously established a defucosylated mouse-dog chimeric anti-EGFR mAb (E134Bf) and a mouse-dog chimeric anti-HER2 mAb (H77Bf), which exerted antitumor activities in canine tumor xenograft models. Here, we produced E134Bf antibody fused to H77Bf single chain Fv at the light chains (E134Bf-H77scFv). The bispecific E134Bf-H77scFv recognized dog EGFR (dEGFR) and dog HER2 (dHER2)-overexpressed Chinese hamster ovary-K1 cells by flow cytometry. E134Bf-H77scFv also reacted with dEGFR and dHER2-positive canine osteosarcoma D-17 cells, and possesses a high binding-affinity ($K_D$: $1.3 \times 10^{-9}$ M). Furthermore, E134Bf-H77scFv exerted antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity against D-17 cells in the presence of canine mononuclear cells and complement, respectively. Moreover, administration of E134Bf-H77scFv suppressed the development of D-17 xenograft tumor in mice early compared with the control dog IgG, E134Bf and H77Bf alone. These results indicate that E134Bf-H77scFv exerts antitumor activities against dEGFR/dHER2-positive canine tumors, and could be a valuable treatment regimen for canine tumors.

**Keywords:** EGFR; HER2; bispecific antibody; ADCC; CDC; canine osteosarcoma

## 1. Introduction

Therapeutic monoclonal antibodies (mAbs) for tumors have revolutionized over several decades due to the development of the hybridoma technique [1] and the mAb humanization technique [2]. Currently, more than 100 mAbs have been approved by the US Food and Drug Administration (FDA) for the treatment of not only tumors, but also inflammatory diseases [3]. MAbs can bind to target antigens with high specificity, and

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inhibit tumor promoting growth factor signaling pathways through their neutralization effects. In addition, mAbs potentiate the host immune responses including antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP) [4]. Due to the successful application of immunoglobulin G (IgG) type mAbs, several antibody formats including bispecific antibodies and antibody–drug conjugates (ADCs) have been approved by the FDA as next generation therapeutic agents for various types of tumors [5,6].

Targeting receptor tyrosine kinase signaling pathways is one of the important strategies for tumor therapy [7]. Human epidermal growth factor receptor (HER) families belong to type I transmembrane glycoproteins that are overexpressed in various solid tumors [8]. Through their homo- and hetero-dimerization, a broad repertoire of oncogenic signaling pathways is activated, and these are essential for tumor development [9]. Anti-epidermal growth factor receptor (EGFR) mAb, cetuximab, can block EGFR activation through the binding to its ligand binding domain and, thus, inhibit the EGFR signaling pathways. Moreover, cetuximab ($IgG_1$ isotype) exhibits ADCC activity, and directs cytotoxic immune effector cells to EGFR-positive tumor cells [10,11]. Cetuximab was approved in combination with platinum-based chemotherapy, showing good outcomes including overall survival, response rate, and progression-free survival in metastatic/recurrent head and neck squamous cell carcinomas [12]. Trastuzumab, the first FDA-approved anti-HER2 mAb, has been the most effective therapy for HER2-positive breast cancers and later in HER2-positive gastric cancers for more than 20 years [13]. Clinically, the efficacy is mediated by the involvement of immunologic engagements, including ADCC, CDC, and ADCP activity [4].

With the increase in lifespan of both humans and dogs, the tumor incidence has increased, as well. Spontaneous canine tumors closely look like human tumors [14]. Osteosarcoma (OS) is the most common primary bone tumor in dogs. The rate of OS in dogs is 27 times higher than that in humans, and lung metastasis is frequently observed. In fact, a one-year survival rate is only 45% [15]. Currently, treatment options for OS in dogs include surgery, radiation, and chemotherapy [16,17]. However, sufficient therapeutic efficacies have not been reported. Therefore, the development of other therapeutic options for canine OS is necessary. Specifically, EGFR expression was significantly upregulated in OS lung metastasis compared to the extrapulmonary sites [18]. Doyle *et al.* reported the vaccine therapy using dog EGFR (dEGFR)/dog HER2 (dHER2) peptide in canine osteosarcoma. Dogs with osteosarcoma were immunized with a dEGFR/dHER2 peptide vaccine, which resulted in an increased anti-dEGFR/dHER2 antibody production and T cells infiltration into tumors. The vaccinated dogs with osteosarcoma showed tumor regression and increased survival. These data support the approach of amplifying antitumor immunity by dEGFR/dHER2 peptide [19].

The ADCC is activated by the binding of the FcγRIIIa on natural killer (NK) cells to the Fc region of mAbs [4]. However, the *N*-linked glycosylation in the Fc region is reported to reduce the binding to the FcγRIIIa on NK cells [20,21]. Fucosyltransferase 8 (FUT8) is an important enzyme that mediates the *N*-linked glycosylation (core fucosylation) in the Fc region [21]. Therefore, FUT8-knockout Chinese hamster ovary (CHO) cells have been shown to produce completely defucosylated recombinant antibodies. The defucosylated mAb strongly binds to FcγRIIIa and exerts potent ADCC activity when compared to a conventional mAb [22].

Previously, we developed an anti-EGFR mAb (EMab-134) [23] and anti-HER2 mAb ($H_2$Mab-77) [24] using the Cell-Based Immunization and Screening method. Subsequently, we produced mouse-dog chimeric anti-EGFR mAb (E134B) and anti-HER2 mAb (H77B) from the information of variable regions of EMab-134 and $H_2$Mab-77, respectively. Furthermore, we evaluated the antitumor effect of their respective defucosylated forms, E134Bf [25,26] and H77Bf [27] against canine tumors in mouse xenograft models. In this study, we produced a defucosylated bispecific Ab against dEGFR and dHER2, and evaluated the ADCC, CDC, and antitumor activities in canine osteosarcoma D-17 xenograft.

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## 2. Materials and Methods

### *2.1 Cell lines*

CHO-K1 and a canine OS cell line D-17 were purchased from the American Type Culture Collection. Stable dog EGFR-overexpressed CHO-K1 (CHO/dEGFR) [26] and dog HER2-overexpressed CHO-K1 (CHO/dHER2) [27] were established as described previously. CHO-K1, CHO/dEGFR, and CHO/dHER2 were cultured in RPMI-1640 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.). D-17 was cultured in MEM medium (Nacalai Tesque, Inc.), supplemented with 10% FBS, 1 mM of sodium pyruvate, 100 units/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B.

### *2.2 Animals*

Following regulations and guidelines to minimize animal distress and suffering in the laboratory, animal experiments for the potential antitumor activity of E134Bf-H77scFv were performed (Approval No. 2022-024) by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (Numazu, Japan). Mice were maintained on an 11 h light/13 h dark cycle in a specific pathogen-free environment throughout the experimental period. Food and water were supplied *ad libitum.* The weight and health of the experimental animals were monitored every 2–5 days during the experiments. The loss of original body weight was determined to a point more than 25% and/or a maximum tumor size >3,000 $mm^3$ as humane endpoints for euthanasia.

### *2.3 Antibodies*

Previously, we have established mouse-dog chimeric anti-EGFR mAb (E134B) [26] and anti-HER2 mAb (H77B) [27]. In this study, to generate E134Bf-H77scFv, we first constructed a single chain Fv of H77B (H77scFv) by connecting the $V_H$ and $V_L$ cDNA of H77B with a linker sequence (GGGGSGGGGSGGGGS). The H77scFv cDNA was further fused at the 3′ end of the light chain cDNA of E134B. The cDNA of the E134B heavy chain and E134B light chain-H77scFv were transduced into BINDS-09 (FUT8 knockout ExpiCHO-S) cells using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.) to produce the defucosylated form [25-32]. A defucosylated and bispecific antibody, E134Bf-H77scFv, was purified using Ab-Capcher (ProteNova Co., Ltd.). We confirmed their purity by SDS-PAGE. Dog IgG was purchased from Jackson ImmunoResearch Laboratories, Inc.

### *2.4 Flow cytometry*

CHO-K1, CHO/dEGFR, CHO/dHER2, and D-17 were obtained by 0.25% trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai Tesque, Inc.) treatment. Cells were treated with E134Bf, H77Bf, E134Bf-H77scFv, or blocking buffer [0.1% bovine serum albumin (BSA; Nacalai Tesque, Inc.) in phosphate-buffered saline (PBS)] (control) for 30 min at 4°C. Subsequently, cells were incubated in FITC-conjugated anti-dog IgG (1:1000; Thermo Fisher Scientific Inc.) for 30 min at 4°C. Fluorescence data were collected and analyzed by the Cell Analyzer EC800 and analyzed by EC800 software ver. 1.3.6 (Sony Corp.).

### 2.5 *Determination of binding affinity*

Serially diluted E134Bf (0.0006-10 μg/mL), H77Bf (0.0006-10 μg/mL), and E134Bf-H77scFv (0.0006-10 μg/mL) were suspended with CHO/dEGFR, CHO/dHER2, and D-17 cells. The cells were further treated with FITC-conjugated anti-dog IgG (1:100). Fluorescence data were obtained by the Cell Analyzer EC800. To determine the dissociation constant ($K_D$),

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GraphPad Prism version 8 (the fitting binding isotherms to built-in one-site binding models, GraphPad Software, Inc.) was used.

*2.6 ADCC*

A ADCC induction by E134Bf, H77Bf, and E134Bf-H77scFv was assayed as follows. Canine mononuclear cells (MNCs) obtained from Yamaguchi University were resuspended in DMEM (Nacalai Tesque, Inc.) with 10% FBS and were used as effector cells. Target D-17 cells were labeled with 10 μg/mL Calcein AM (Thermo Fisher Scientific, Inc.) [25-42]. The target cells ($2 \times 10^4$ cells) were plated in 96-well plates and mixed with effector canine MNCs (effector/target cells ratio, 50), 100 μg/mL of control normal dog IgG, E134Bf, H77Bf, and E134Bf-H77scFv. Following incubation for 4.5 h at 37°C, the release of calcein into the culture medium was analyzed using a microplate reader (Power Scan HT; BioTek Instruments, Inc.) with an excitation wavelength (485 nm) and an emission wavelength (538 nm). Cytolyticity (% lysis) was calculated as follows: percent lysis = (E − S)/(M − S) × 100, where "E" is the fluorescence in cultures of both effector and target cells, "−S" is the spontaneous fluorescence of only target cells, and "M" is the maximum fluorescence following the treatment with a lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM of EDTA, and 0.5% Triton X-100).

*2.7 CDC*

Target D-17 cells were treated with 10 μg/mL calcein AM [25-42]. The target cells ($2 \times 10^4$ cells) were mixed with rabbit complement (final dilution 1:10; Low-Tox-M Rabbit Complement; Cedarlane Laboratories) and 100 μg/mL of control normal dog IgG, E134Bf, H77Bf, and E134Bf-H77scFv. Following incubation for 4.5 h at 37°C, the release of calcein into the medium was determined.

2.8 *Antitumor activities in xenografts of D-17*

BALB/c nude mice (female, 4 weeks old) were purchased from Charles River Laboratories, Inc. D-17 cells ($5 \times 10^6$ cells) suspended with BD Matrigel Matrix Growth Factor Reduced (BD Biosciences) were subcutaneously inoculated into the left flank of mice. On day 8 after the inoculation, 100 μg of E134Bf, H77Bf, and E134Bf-H77scFv, and control normal dog IgG (n = 8) in 100 μL PBS were injected intraperitoneally. Additional antibody injections were performed on days 15 and 22. Furthermore, on days 8, 15, and 22, canine MNCs ($5 \times 10^5$ cells) were injected into the surrounding tumors. The tumor volume was measured on days 8, 10, 15, 17, 22, and 25, and determined, as described previously [25-43].

**3. Results**

*3.1. Flow cytometric analysis against dEGFR and dHER2-expressing cells using E134Bf-H77scFv*

We previously developed mouse-dog chimeric mAbs including E134B (anti-dEGFR) and H77B (anti-dHER2), and also produced the corresponding defucosylated forms, E134Bf [26] and H77Bf [27], respectively. To generate a bispecific Ab against dEGFR and dHER2, we first constructed the cDNA of H77scFv, following the ligation to the light chain cDNA of E134B (Fig. 1). Subsequently, we transduced the cDNAs of E134B heavy chain and the E134B light chain-H77scFv into FUT8 knockout ExpiCHO-S cells, and purified the defucosylated bispecific Ab against dEGFR and dHER2, which is labeled as E134Bf-H77scFv.

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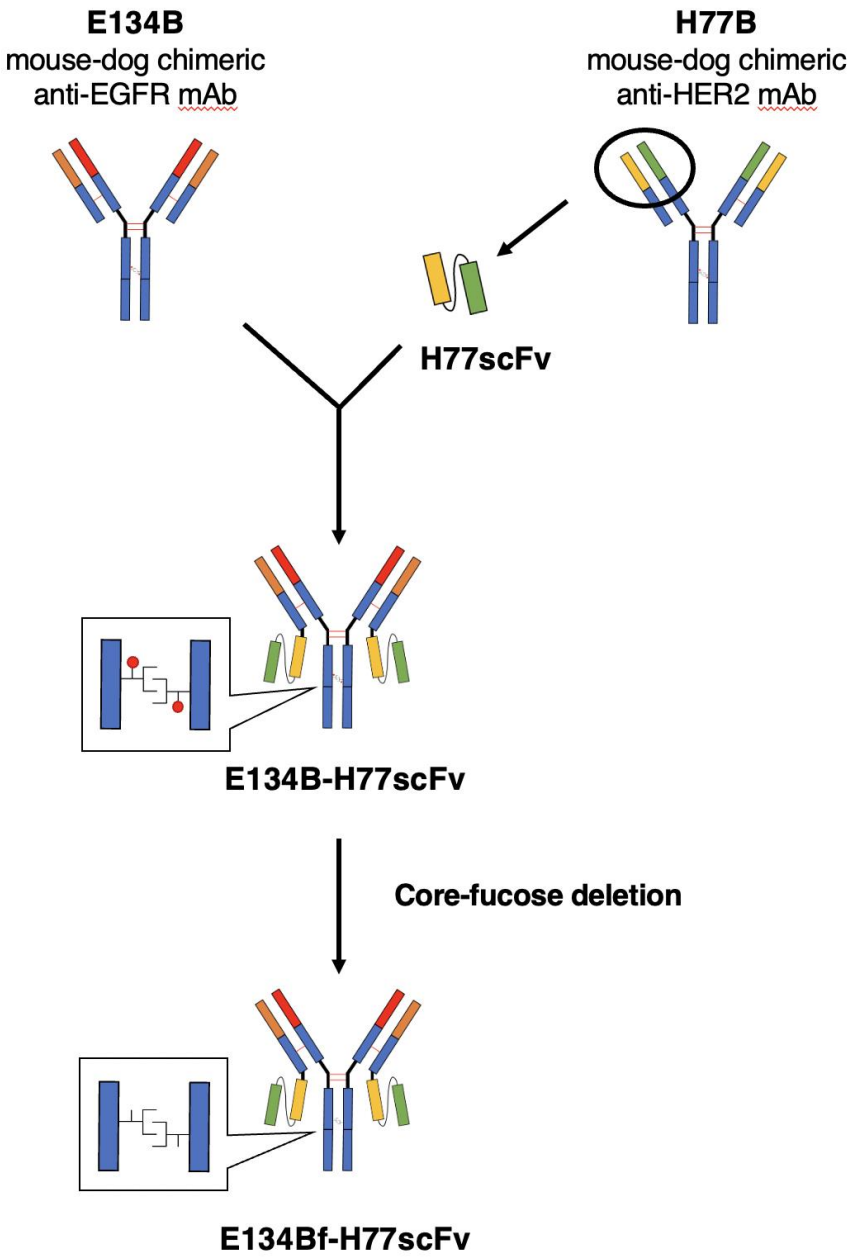


**Figure 1.** Schematic illustration of the production of bispecific antibody, E134Bf-H77scFv from E134B and H77B.

We first confirmed the reactivity of E134Bf-H77scFv to dEGFR and dHER2 expressed cells using flow cytometry. As shown in Fig. 2, E134Bf-H77scFv exhibited the similar reactivities to CHO/dEGFR (Fig. 2A) and CHO/dHER2 (Fig. 2B) cells compared with E134Bf and H77Bf, respectively. In contrast, E134Bf-H77scFv never reacted with parental CHO-K1 cells (Fig. 2C). Thereafter, we examined the reactivity of E134Bf-H77scFv against dEGFR and dHER2-positive canine osteosarcoma D-17 cells. As shown in Fig. 2D, E134Bf-H77scFv could recognize D-17 cells. Then, we performed a kinetic analysis of the interactions of E134Bf, H77Bf and E134Bf-H77scFv with D-17 via flow cytometry. As shown in Fig. 3, the $K_D$ values for the interaction of E134Bf, H77Bf, and E134Bf-H77scFv with D-17 cells were $6.0 \times 10^{-10}$ M, $2.9 \times 10^{-10}$ M, and $1.3 \times 10^{-9}$ M, respectively.

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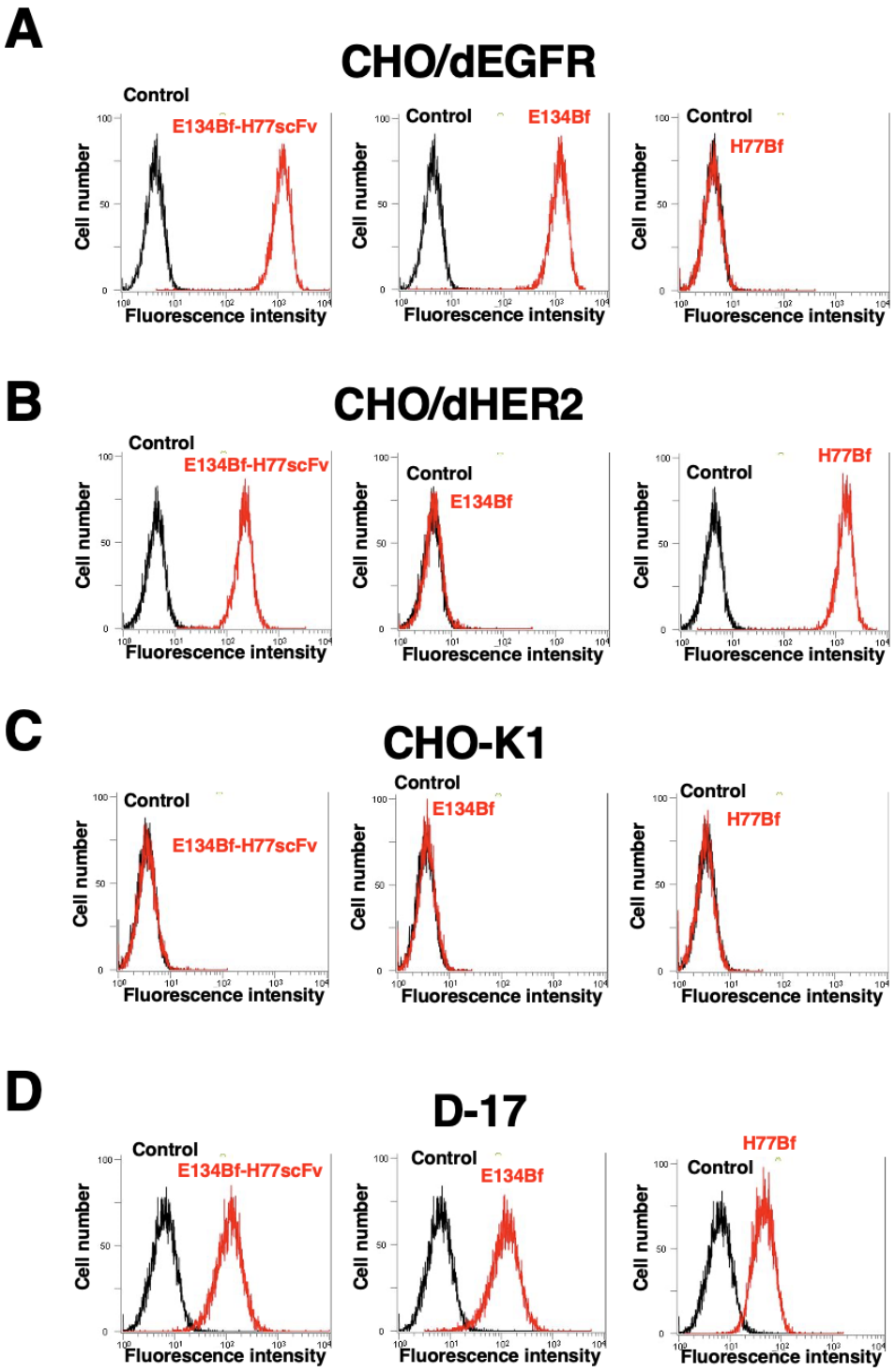


**Figure 2.** Flow cytometry using E134Bf, H77Bf, and E134Bf-H77scFv. CHO/dEGFR (A), CHO/dHER2 (B), CHO-K1 (C), and D-17 (D) cells were treated with E134Bf, H77Bf, and E134Bf-H77scFv or buffer control, followed by FITC-conjugated anti-dog IgG. Fluorescence data were analyzed using the Cell Analyzer EC800.

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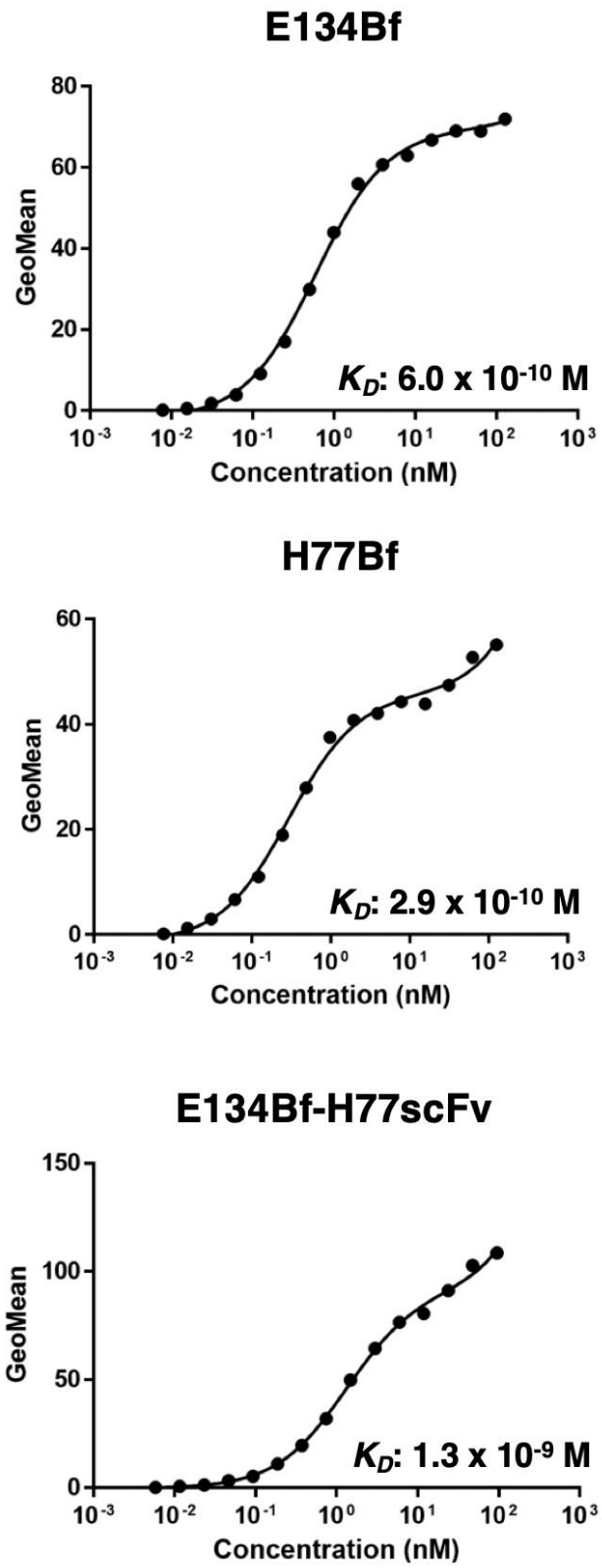


**Figure 3.** The determination of the binding affinity of E134Bf, H77Bf, and E134Bf-H77scFv for D-17 cells using flow cytometry. D-17 cells were suspended in serially diluted E134Bf, H77Bf, and E134Bf-H77scFv, followed by the addition of FITC-conjugated anti-dog IgG. Fluorescence data were analyzed using the Cell Analyzer EC800, following the calculation of the dissociation constant ($K_D$) by GraphPad PRISM 8.

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*3.2. E134Bf-H77scFv-mediated ADCC and CDC in D-17 cells*

We next investigated whether E134Bf-H77scFv was capable of mediating ADCC against D-17 cells. E134Bf-H77scFv showed ADCC (15.2% cytotoxicity) against D-17 cells more effectively than the control normal dog IgG (4.1 % cytotoxicity; $P < 0.01$). There was no significant difference between E134Bf or H77Bf and the control normal dog IgG against D-17 in this experimental condition (Fig. 4A). We next examined whether E134Bf-H77scFv could exert CDC against D-17 cells. As shown in Fig. 4B, E134Bf, H77Bf, and E134Bf-H77scFv induced a higher degree of CDC (33.6% [$P < 0.05$], 40.7% [$P < 0.01$], and 44.4% [$P < 0.01$] cytotoxicity, respectively) in D-17 cells compared with that induced by the control normal dog IgG (13.8% cytotoxicity). These results indicated that E134Bf-H77scFv exhibited higher levels of ADCC and CDC activities against D-17 cells.

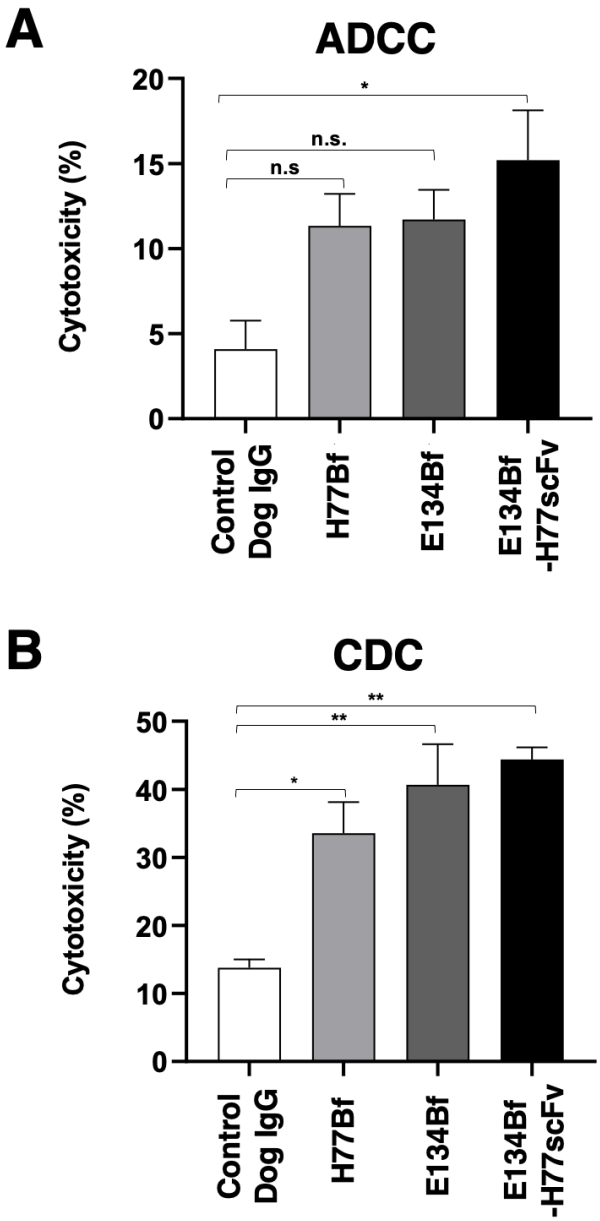


**Figure 4.** Evaluation of ADCC and CDC elicited by E134Bf, H77Bf, and E134Bf-H77scFv. (A) ADCC elicited by E134Bf, H77Bf, E134Bf-H77scFv, and control dog IgG targeting D-17 cells. (B) CDC elicited by E134Bf, H77Bf, E134Bf-H77scFv, and control dog IgG targeting D-17 cells. Values are presented as the mean ± SEM. ($^{**}P < 0.01$ and $^{*}P < 0.05$; Welch's *t* test). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; n.s., not significant.

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*3.3. Antitumor effects of E134Bf-H77scFv in the mouse xenograft of D-17 cells*

In the D-17 xenograft tumor, E134Bf-H77scFv, E134Bf, H77Bf and control normal dog IgG were intraperitoneally injected into mice on days 8 , 15, and 22 after D-17 cell inoculation. Furthermore, on days 8, 15, and 22, canine MNCs were injected surrounding the tumors. On days 8, 10, 15, 17, 22, and 25 after the inoculation, the tumor volume was measured. The E134Bf-H77scFv administration resulted in faster reduction of tumor volume on days 10 ($P < 0.05$), 15 ($P < 0.01$), and 17 ($P < 0.01$) than that of the E134Bf, H77Bf and control normal dog IgG (Fig. 5A). However, on days 22 and 25, significant reduction of tumor was also observed in E134Bf and H77Bf treated groups as well as the E134Bf-H77scFv treated group compared with the controls (Fig. 5A). As shown in Fig. 5B, the weight of D-17 tumors treated with E134Bf-H77scFv, E134Bf, and H77Bf was significantly lower than that treated with control normal dog IgG (70%, 64%, 58% reduction, respectively; $P<0.01$). D-17 tumors that were resected from mice on day 25 are shown in Fig. 5C. The loss of body weight was not observed in each group (Fig. 5D). The mice on day 25 are shown in Fig. 5E.

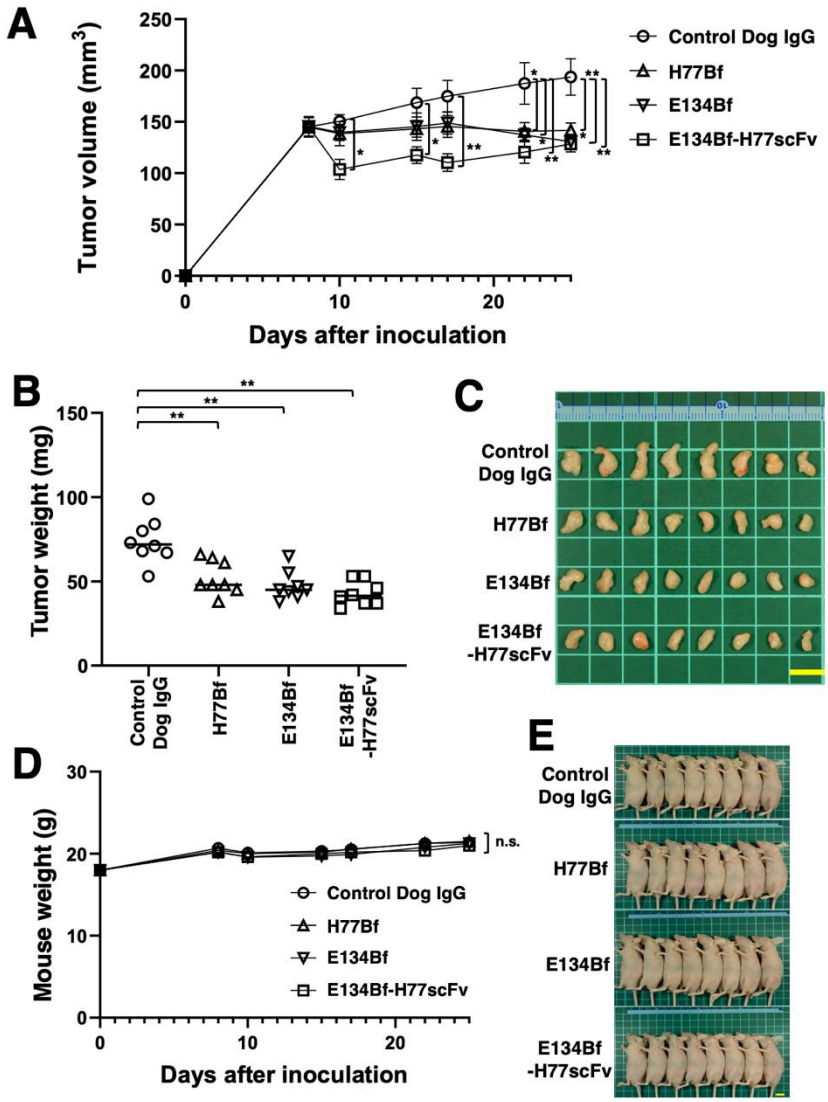


**Figure 5.** Antitumor activity of E134Bf, H77Bf, and E134Bf-H77scFv. (A) Evaluation of tumor volumes in D-17 xenograft models. D-17 cells ($5 \times 10^6$ cells) were subcutaneously injected into mice. On day 8, 100 μg of E134Bf, H77Bf, E134Bf-H77scFv or control dog IgG were injected intraperitoneally into mice. Additional antibodies were injected on days 15 and 22. Mononuclear cells were also injected surrounding the tumors on days 8, 15, and 22. The tumor volume was measured on days 8, 10, 15, 17, 22, and 25 after the injection. Values are presented as the mean ± SEM. $^{**}P < 0.01$ and $^{*}P <$

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0.05 (ANOVA and Sidak's multiple comparisons test). (B) Tumor weight (day 25) was measured from excised xenografts. Values are presented as the mean ± SEM. $^{**}P < 0.01$ (Welch's *t* test). (C) Appearance of resected tumors from the indicated groups on day 25 (scale bar, 1 cm). (D) Body weights of mice implanted with D-17 xenografts on days 8, 10, 15, 17, 22, and 25 (ANOVA and Sidak's multiple comparisons test). n.s., not significant. (E) Body appearance of D-17-implanted mice on day 25 (scale bar, 1 cm).

## 4. Discussion

In this study, we developed a novel bispecific antibody E134Bf-H77scFv against dEGFR and dHER2 (Fig. 1), and showed more potent ADCC activity compared with that of E134Bf and H77Bf (Fig. 4A). Furthermore, E134Bf-H77scFv showed the antitumor activity against D-17 xenografts at earlier periods compared with E134Bf and H77Bf treatments (Fig. 5A), suggesting that E134Bf-H77scFv possesses the different mode of actions. As shown in Fig. 2, E134Bf-H77scFv recognized dEGFR and dHER2-overexpressed CHO-K1 cells. Furthermore, E134Bf-H77scFv also reacted with dEGFR and dHER2 double positive D-17 cells. However, we have not determined whether E134Bf-H77scFv can induce the cross-link between dEGFR and dHER2 on the cell surface. In our epitope mapping analysis, EMab-134, the original mAb of E134B, recognized the sequence ($_{377}$-RGDSFTHTPP-$_{386}$) in domain III of EGFR [44]. However, the epitope of H77scFv has not been identified. Further investigations are essential to reveal the structural relevance of recognition, and the mechanism of the antitumor activity.

E134Bf-H77scFv is a novel modality for targeting dEGFR and dHER2. Previously, different types of dual-targeting EGFR and HER2 bispecific modalities have been tested in preclinical studies. A bispecific affibody molecule to EGFR or HER2 was generated by linking a bivalent HER2-binding affibody to a bivalent EGFR-binding affibody with a linker sequence (Gly4-Ser)3. The bispecific affibody was shown to bind to both HER2-overexpressed SKBR-3 and EGFR-overexpressed A-431 cells [45]. "AffiMabs" were generated by fusing an EGFR-targeting affibody molecule to trastuzumab's heavy or light chains. AffiMabs was shown to induce the downregulation of both EGFR and HER2. Furthermore, AffiMabs exhibited a more potent anti-proliferative effect than trastuzumab i*n vitro* [46]. Moreover, a bispecific nanobody targeting EGFR and HER2 (bsNb) was developed. The bsNb was further ligated to rhamnose (Rha) hapten to reconstitute the Fc effector biological function. The bsNb-Rha retained dual-targeting activity to EGFR and HER2, and elicited potent antitumor effect *in vivo* via the Fc-mediated engagement of endogenous anti-Rha antibodies [47]. However, bispecific antibody formats against EGFR and HER2 have not been applied in the clinic at present. Studies have demonstrated that overexpression of EGFR family proteins is observed in aggressive canine tumors including osteosarcoma [48] and hemangiosarcoma [49] with shortened the survival. We have already showed that EMab-134 and $H_2$Mab-77, original antibodies of E134Bf-H77scFv, can be used in immunohistochemistry [23,24]. Therefore, EMab-134, $H_2$Mab-77, and E134Bf-H77scFv are expected to be used in clinics for both the diagnosis and treatment of canine tumors.

Bispecific antibodies have been approved as therapeutic agents to cover unmet clinical needs [50,51]. A wide range of bispecific antibody formats has been developed for cancer therapy through different mechanisms including the engagement of T cells or other immune cells (e.g., NK cells) to induce antitumor immunity, and bridging receptors to inhibit or activate the downstream signaling pathways [52]. For targeting receptor kinase signaling pathways including EGFR, HER2, HER3, and MET in tumor cells, GBR1302 has been developed as a T-cell engager to direct CD3-positive T cells to HER2 on tumor cells [53]. Furthermore, bispecific antibodies that recognize different receptors involved in signaling crosstalk can be used to avoid bypass signal transduction during tumor development [54-56]. Growing number of evidence has indicated that Met signaling contributes to the resistance of EGFR tyrosine kinase inhibitors in non-small cell lung cancers (NSCLC) [57-59]. Therefore, dual inhibition of aberrant oncogenic signaling has become a promising strategy. Amivantamab is a dual-targeting EGFR and MET bispecific antibody that can inhibit the EGFR and MET signaling pathways [56]. In various tumor models,

amivantamab efficiently downregulates the expression of EGFR and MET, and exhibited antitumor immunity with Fc-mediated effector interactions [60]. The FDA granted accelerated approval to JNJ-61186372 (Amivantamab) for the treatment for locally advanced or metastatic NSCLC. Another example is MCLA-128, a bispecific antibody for HER2 and HER3. MCLA-128 can inhibit heregulin (a HER3 ligand)-mediated signaling of HER2/HER3, and suppress tumor cell survival and proliferation via the downregulation of PI3K/Akt signaling [61]. Clinical studies on MCLA-128 are ongoing in patients with breast cancer, pancreatic cancer, and NSCLC.

For the development of E134Bf-H77scFv to treat canine tumors in the clinic, the effect of E134Bf-H77scFv on dEGFR homodimer and dEGFR/dHER2 heterodimer-mediated signaling should be investigated. Further, it should be determined whether E134Bf-H77scFv can induce the internalization of dEGFR and dHER2. Canine tumors represent an outbred population, and resemble human tumors in the initiation, disease progression, growth factors and oncogene mutations. Therefore, future investigations could contribute to not only canine, but also human tumor treatment.

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