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Defucosylated Anti-Epidermal Growth Factor Receptor Monoclonal Antibody (134-mG_{2a}-f) Exerts Antitumor Activities in Mouse Xenograft Models of Canine Osteosarcoma

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The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein. Although EGFR is physiologically essential in normal cells, it contributes to tumor malignancy through gene amplification and/or protein overexpression, which augment signaling cascades in tumor cells. We previously developed an anti-human EGFR (hEGFR) monoclonal antibody (mAb), EMab-134 (mouse IgG_1, kappa), which detects hEGFR and dog EGFR (dEGFR) with high sensitivity and specificity. The mouse IgG_{2a} version of EMab-134 (134-mG_{2a}) has antitumor effects toward mouse xenografts of hEGFR-expressing oral squamous cell carcinomas. Furthermore, 134-mG_{2a}-f, the defucosylated version of 134-mG_{2a}, 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. Herein, the reactivity of 134-mG_{2a}-f against canine cancer cells with endogenous dEGFR was first examined by flow cytometry and immunocytochemistry. *In vitro* analysis demonstrated that 134-mG_{2a}-f highly exerted ADCC and CDC for a canine osteosarcoma cell line, D-17, which expresses endogenous dEGFR. Moreover, *in vivo* administration of 134-mG_{2a}-f significantly suppressed the development of D-17 compared with the results in response to control mouse IgG. These results suggest that 134-mG_{2a}-f exerts antitumor effects against dEGFR-expressing canine cancers, and could be valuable as part of an antibody treatment regimen for them.

Keywords: EGFR, monoclonal antibody, ADCC, CDC, canine osteosarcoma, antitumor activity

Introduction

The epidermal growth factor receptor (EGFR) is a type-I transmembrane glycoprotein and a receptor tyrosine kinase.[1,2] EGFR can form homodimers or heterodimers with other EGFR family members, including HER2 (ErbB2/neu), HER3 (ErbB3), and HER4 (ErbB4). These dimers activate various signaling pathways, including Ras/Raf/MEK/ERK, PI3K-AKT-mTOR, and JAK-STAT pathways.[3] These pathways promote cell proliferation and migration, and inhibit apoptosis. EGFR gene mutation is most common in lung cancer,[4] and overexpression is observed in many tumors, such as osteosarcoma (OS),[5,6] glioblastoma,[7] breast cancer,[8] lung cancer,[4] and colorectal cancer,[9] which contributes to tumor malignancy by augmenting the above signaling pathways.

OS is the most common primary bone tumor in dogs, leading to metastasis.[10] The incidence of OS in dogs is 27 times higher than that in humans, with a 1-year survival rate of only about 45%.[11] Although therapy options for canine

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OS include surgery (limb amputation or limb-sparing surgery), radiotherapy, and chemotherapy,[12,13] they are not sufficiently effective. Therefore, the development of therapeutic strategies for canine OS is still necessary.

Previously, we developed a novel anti-human EGFR (hEGFR) monoclonal antibody (mAb), EMab-134 (mouse IgG_1, kappa), by immunizing mice with the purified recombinant ectodomain of hEGFR (hEGFRec) from culture supernatants of hEGFRec-overexpressed human glioblastoma LN229 cells.[14] EMab-134 can be used in flow cytometry, Western blotting, and immunohistochemical analyses. The mouse IgG_{2a} version of EMab-134 (134-mG_{2a}) exerts antitumor activities in mouse xenograft models of hEGFR-expressing oral squamous cell carcinoma.[15] In addition, we produced the defucosylated version of 134-mG_{2a}, 134-mG_{2a}-f, to augment antibody-dependent cellular cytotoxicity (ADCC).[16] 134-mG_{2a}-f exhibits ADCC and complement-dependent cytotoxicity (CDC) in dog EGFR (dEGFR)-overexpressed CHO-K1 (CHO/dEGFR) cells and antitumor activities in mouse xenograft models of CHO/dEGFR cells.[16] In the present study, we investigated antitumor activities of 134-mG_{2a}-f against the D-17 canine tumor cell line endogenously expressed dEGFR.

Materials and Methods

Antibodies

Anti-hEGFR mAb EMab-134 (mouse IgG_1, kappa) was developed as previously described.[14] To generate recombinant EMab-134 (recEMab-134), we subcloned V_H and C_H of cDNAs of EMab-134 into the pCAG-Neo vector, along with V_L and C_L cDNAs of EMab-134 into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. To generate 134-mG_{2a}, we subcloned V_H cDNA of EMab-134 and C_H mouse IgG_{2a} into the pCAG-Neo vector, along with V_L and C_L cDNAs of EMab-134 into the pCAG-Ble vector, respectively.[15] Vectors of EMab-134 and 134-mG_{2a} were transfected into ExpiCHO-S cells and BINDS-09 cells (FUT8-deficient ExpiCHO-S cells) using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc., Waltham, MA), respectively.[16]

The resulting mAbs, recEMab-134 and 134-mG_{2a}-f, were purified with Protein G-Sepharose (GE Healthcare Biosciences, Pittsburgh, PA).[16] Mouse IgG (Cat. No. I8765) and IgG_{2a} (Cat. No. M7769) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines

A canine OS cell line, D-17, was obtained from the American Type Culture Collection (Manassas, VA). D-17 was cultured in MEM (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 1 mM of sodium pyruvate, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Animals

All animal experiments were performed following relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments for ADCC and antitumor activity were approved by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (Permit Nos. 2021-028 for ADCC assays and 2021-021 for antitumor experiments). Mice were maintained in a specific pathogen-free environment (23°C ± 2°C, 55% ± 5% humidity) on an 11-hour light/13-hour 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 maximum tumor size >3000 mm^3 as humane endpoints for euthanasia. Mice were euthanized by cervical dislocation; death was verified by respiratory and cardiac arrest.

Flow cytometry

D-17 cells were harvested by brief exposure to 0.25% trypsin/1 mM ethylenediaminetetraaceticacid (EDTA; Nacalai Tesque, Inc.). After washing with blocking buffer of 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), cells were treated with 1 μg/mL of recEMab-134, 134-mG_{2a}-f, or control blocking buffer for 30 minutes at 4°C. Then, cells were incubated in Alexa Fluor 488-conjugated anti-mouse IgG at a dilution of 1:1000 (Cat No. 4408S; Cell Signaling Technology, Inc., Danvers, MA) for 30 minutes at 4°C. Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Immunocytochemical analysis

D-17 cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, quenched with 50 mM NH_4Cl in PBSc/m (PBS supplemented with 0.2 mM Ca^{2+} and 2 mM Mg^{2+}) for 10 minutes, and treated with blocking buffer (PBSc/m supplemented with 0.5% BSA) for 30 minutes. Then, the cells were incubated with 10 μg/mL of recEMab-134 or 134-mG_{2a}-f or control blocking buffer for 1 hour followed by Alexa Fluor 488-conjugated anti-mouse IgG (1:400) and DAPI (Thermo Fisher Scientific, Inc.) for 45 minutes. Fluorescence images were acquired with a 40× objective on a fluorescence microscope BZ-X800 (Keyence, Osaka, Japan).

Determination of binding affinity

D-17 cells were suspended in 100 μL of serially diluted 134-mG_{2a}-f (0.006–100 μg/mL) followed by Alexa Fluor 488-conjugated anti-mouse 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).

Antibody-dependent cellular cytotoxicity

A total of five female 5-week-old BALB/c nude mice (weighing 14–17 g) were purchased from Charles River Laboratories, Inc. (Kanagawa, Japan). Spleen cells from five mice were used as the source of NK cells for the evaluation of ADCC, as previously reported.[16] Following euthanasia by cervical dislocation, the spleens were removed aseptically, and a syringe was used to force spleen tissues through a sterile cell strainer (352360; BD Falcon, Corning, New York, NY) and obtain single-cell suspensions. Erythrocytes were lysed by a 10-second exposure to ice-cold distilled water.

Splenocytes were washed with Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc.) and resuspended in DMEM with 10% FBS to be used as effector cells. Target tumor cells were labeled with 10 μg/mL Calcein AM (Thermo Fisher Scientific, Inc.) and resuspended in the same medium. The target cells (2×10^4 cells/well) were plated in 96-well plates and mixed with effector cells (effector/target cell ratio, 50), 100 μg/mL of 134-mG_{2a}-f, or control mouse IgG_{2a}.

Following a 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 = $(E-S)/(M-S)\times100$, 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.

Complement-dependent cytotoxicity

Target tumor cells were labeled with 10 μg/mL of Calcein AM and resuspended in the medium. They were then plated in 96-well plates at 2×10^4 cells/well with rabbit complement (final dilution 1:10, Low-Tox-M Rabbit Complement; Cedarlane Laboratories, Hornby, Ontario, Canada) and 100 μg/mL of 134-mG_{2a}-f or control mouse IgG_{2a}. 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 Antibody-Dependent Cellular Cytotoxicity section above.

Antitumor activity of 134-mG_{2a}-f in xenografts of D-17 cells

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

On day 7 postinoculation, 100 μg of 134-mG_{2a}-f ($n=8$) or control mouse IgG ($n=8$) in 100 µL of PBS was injected intraperitoneally. Additional antibody inoculations were performed on days 11 and 18. At 25 days following cell implantation, all mice were euthanized by cervical dislocation. Tumor diameters and volumes were determined as previously described.[16]

Statistical analysis

All data are expressed as mean ± 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. ANOVA and Sidak's multiple comparisons tests were conducted for tumor volume and mouse weight. All calculations were performed using GraphPad Prism 8. A *p*-value of <0.05 was considered statistically significant.

Results

Flow cytometry analysis against canine OS D-17 cells using recEMab-134 and 134-mG_{2a}-f

In our previous study, an anti-hEGFR mAb (EMab-134) recognized dEGFR-overexpressed CHO/dEGFR cells, indicating that EMab-134 crossreacts with dEGFR.[16] In this study, recEMab-134 also detected canine OS D-17 cells (Fig. 1A). Similarly, the defucosylated mouse IgG_{2a} type of EMab-134 (134-mG_{2a}-f) detected D-17 cells (Fig. 1B), indicating that both recEMab-134 and 134-mG_{2a}-f could detect the endogenous dEGFR expressed on D-17 cells.

Immunocytochemical analysis against canine OS D-17 cells using recEMab-134 and 134-mG_{2a}-f

We also investigated whether recEMab-134 and 134-mG_{2a}-f were available for immunocytochemistry in D-17 cells. We found that recEMab-134 and 134-mG_{2a}-f, but not buffer control, labeled dEGFR in D-17 cells (Fig. 1C). The result suggests that both recEMab-134 and 134-mG_{2a}-f selectively recognize endogenous dEGFR in D-17 cells.

Determination of binding affinity

A kinetic analysis of the interactions of 134-mG_{2a}-f with D-17 cells was performed via flow cytometry. As shown in Figure 1D, the K_D for the interaction of 134-mG_{2a}-f with D-17 cells was 9.0×10^{-10} M, suggesting that 134-mG_{2a}-f shows high affinity for D-17 cells.

134-mG_{2a}-f-mediated ADCC and CDC in D-17 cells

We investigated whether 134-mG_{2a}-f was capable of mediating ADCC against D-17 cells. As shown in Figure 2A, 134-mG_{2a}-f showed ADCC (14.4% cytotoxicity; $p<0.05$) against D-17 cells more effectively than did the control mouse IgG_{2a} (3.8% cytotoxicity) and the control PBS (2.1% cytotoxicity).

We then investigated whether 134-mG_{2a}-f could mediate CDC against D-17 cells. As shown in Figure 2B, 134-mG_{2a}-f elicited a higher degree of CDC (28.8% cytotoxicity; $p<0.01$) in D-17 cells compared with that elicited by control mouse IgG_{2a} (14.1% cytotoxicity) and the control PBS (8.8% cytotoxicity). These results demonstrated that 134-mG_{2a}-f promoted significantly higher levels of ADCC and CDC against dEGFR-expressing D-17 cells.

Antitumor activities of 134-mG_{2a}-f in the mouse xenografts of D-17 cells

In the D-17 xenograft models, 134-mG_{2a}-f (100 μg) and control mouse IgG (100 μg) were injected intraperitoneally into mice on days 7, 11, and 18, following the injection of D-17 cells. The tumor volume was measured on days 7, 11, 14, 18, 21, and 25 after the injection. The administration of 134-mG_{2a}-f resulted in a significant reduction in tumor development on days 18 ($p<0.01$), 21 ($p<0.01$), and 25 ($p<0.01$) compared with that of the control mouse IgG (Fig. 3A).

The administration of 134-mG_{2a}-f resulted in a 32% reduction of tumor volume compared with that of the control mouse IgG on day 25 postinjection. Furthermore, tumors from the 134-mG_{2a}-f-treated mice weighed significantly less

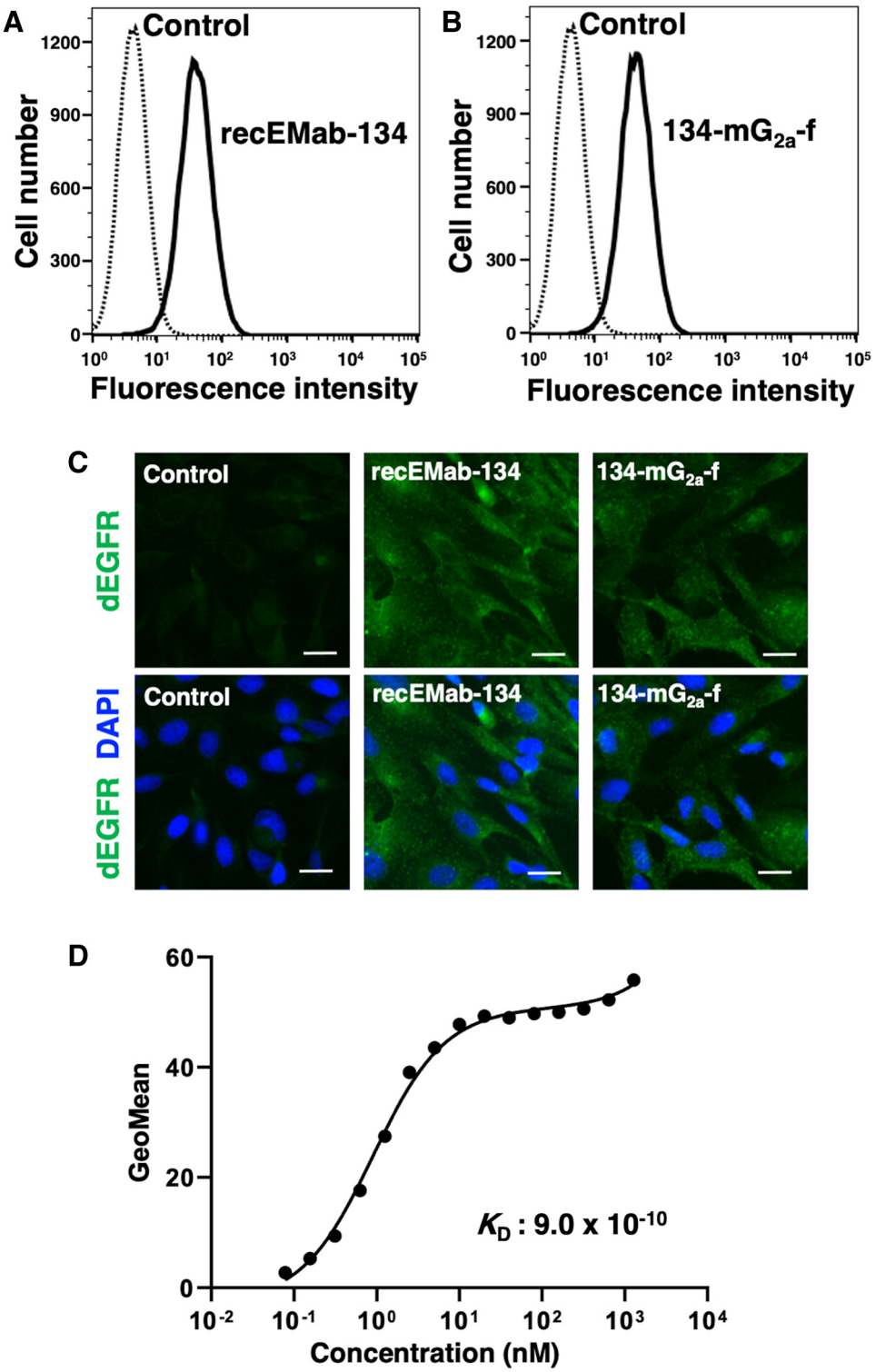

FIG. 1. Flow cytometry and immunocytochemistry using recEMab-134 and 134-mG_{2a}-f. **(A, B)** D-17 cells were treated with recEMab-134 **(A)** and 134-mG_{2a}-f **(B)** or buffer control **(A, B)**, followed by Alexa Fluor 488-conjugated anti-mouse IgG. **(C)** D-17 cells were incubated with buffer control, 10 μg/mL of recEMab-134, or 10 μg/mL of 134-mG_{2a}-f for 1 hour at room temperature. The cells were further incubated with Alexa Fluor 488-conjugated anti-mouse IgG and DAPI for 45 minutes at room temperature. Scale bars, 20 μm. **(D)** Determination of the binding affinity of 134-mG_{2a}-f for D-17 cells using flow cytometry. D-17 cells were suspended in 100 μL of serially diluted 134-mG_{2a}-f, followed by the addition of Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the EC800 Cell Analyzer.

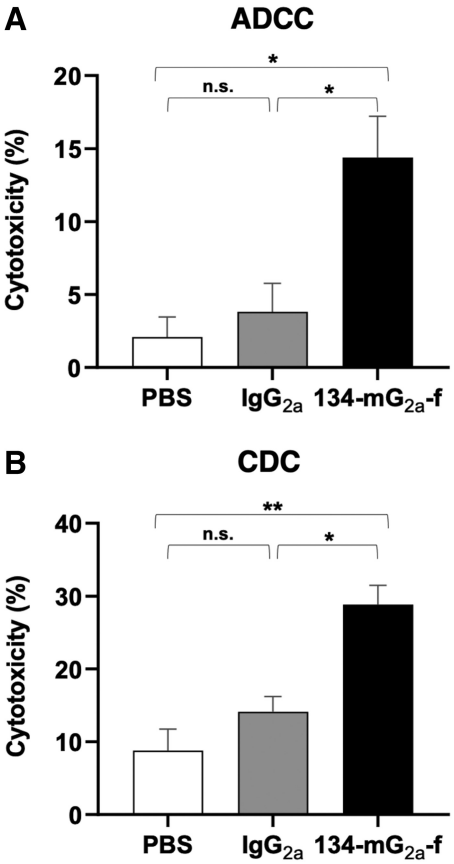

FIG. 2. Evaluation of ADCC and CDC elicited by 134-mG_{2a}-f. **(A)** ADCC elicited by 134-mG_{2a}-f, control mouse IgG_{2a}, or control PBS targeting D-17 cells. Asterisks indicate statistical significance (*$p < 0.05$; Tukey's test). **(B)** CDC elicited by 134-mG_{2a}-f, control mouse IgG_{2a}, or control PBS targeting D-17 cells. Values are mean ± SEM. Asterisks indicate statistical significance (**$p < 0.01$, *$p < 0.05$, 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.

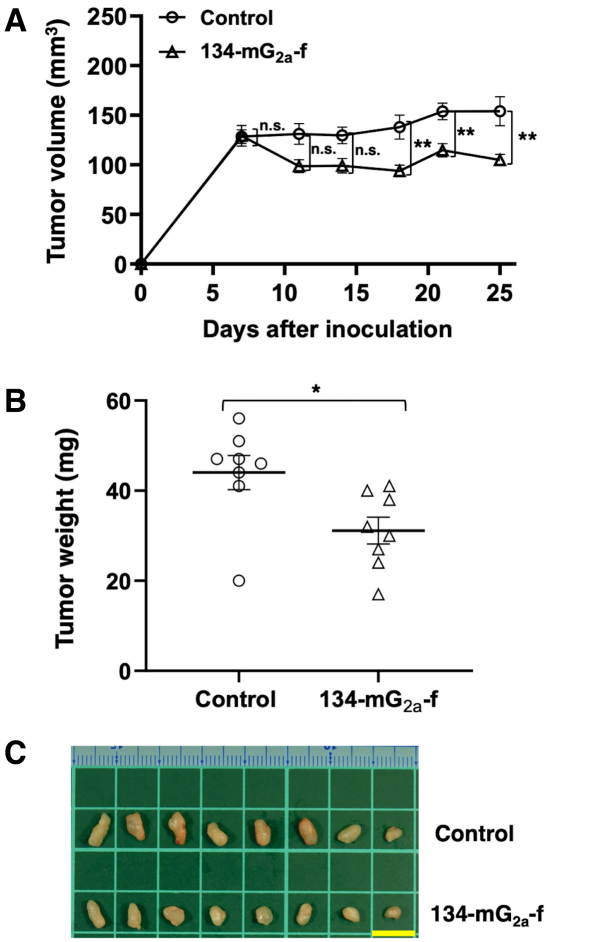

FIG. 3. Evaluation of antitumor activity of 134-mG_{2a}-f in D-17 xenografts. **(A)** D-17 cells (5×10^6 cells) were injected subcutaneously into the left flank. On day 7, 100 μg of 134-mG_{2a}-f or control mouse IgG in 100 μL PBS was injected intraperitoneally into mice; additional antibodies were then injected on days 11 and 18. The tumor volume was measured on days 7, 11, 14, 18, 21, and 25 after the injection. Values are mean ± SEM. Asterisks indicate statistical significance (**$p < 0.01$; ANOVA and Sidak's multiple comparisons test). **(B)** Tumors of D-17 xenografts were resected from 134-mG_{2a}-f and control mouse IgG groups. Tumor weight on day 25 was measured from excised xenografts. Values are mean ± SEM. Asterisk indicates statistical significance (*$p < 0.05$, Welch's *t*-test). **(C)** Resected tumors of D-17 xenografts from the control mouse IgG and 134-mG_{2a}-f groups on day 25. Scale bar, 1 cm.

than those from the control IgG-treated mice (29% reduction; $p < 0.05$, Fig. 3B). Tumors that were resected from mice on day 25 are demonstrated in Figure 3C.

Total body weights did not differ significantly among the two groups (Fig. 4A). The body appearance of mice on day 25 is demonstrated in Figure 4B.

Taken together, these results indicate that the administration of 134-mG_{2a}-f effectively reduced the growth of D-17 xenografts.

Discussion

Cancer is a common cause of death in dogs.[17,18] Among them, canine OS is a highly metastatic and intractable cancer, with 80% of dogs with OS dying from lung metastasis.[10] Canine OS shares various clinical and molecular similarities with human OS, making it a spontaneously occurring large animal disease model that can be used to identify biomarkers and develop treatments.[11] Therefore, developing therapeutic strategies for canine OS will improve not only the clinical response rate of canine OS but also that of human OS.

In canine OS, surgery (limb amputation or limb-sparing surgery) is a first-line treatment. Chemotherapy including carboplatin, cisplatin, and doxorubicin is also used for adjuvant and/or neoadjuvant therapy.[19–22] These treatments have been shown to result in longer survival times than amputation alone.[23] However, these therapies significantly reduce the canine quality of life. Therefore, it is essential to establish a new therapeutic modality.

Because original antibody therapies for dogs have not been established for most diseases including OS, antibody drugs for humans have been used in their place. For example, the hEGFR and dEGFR sequences are 91% identical, and some

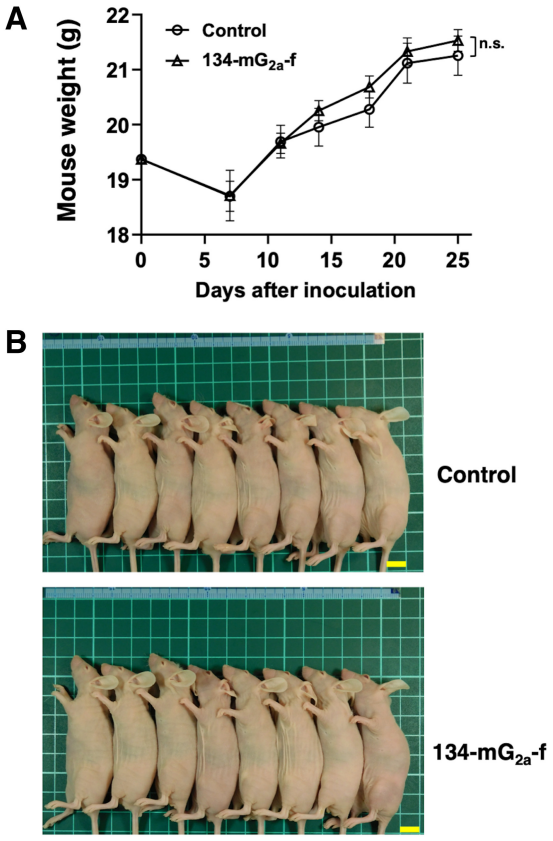

FIG. 4. Body weights and appearance of the mice implanted with D-17 xenografts. **(A)** Body weights of mice implanted with D-17 xenografts were recorded on days 7, 11, 14, 18, 21, and 25. **(B)** Body appearance of mice on day 25. Scale bar, 1 cm.

anti-hEGFR mAbs are effective against canine tumors that overexpress dEGFR *in vitro* and/or *in vivo*.[16,24] D-17 is a canine OS cell line commonly used for various studies.[25–29] Since D-17 cells express EGFR,[30,31] Mantovani *et al.* applied an EGFR-tyrosine kinase inhibitor to suppress D-17 cell proliferation.[30]

However, there is no study to apply an anti-EGFR mAb to EGFR-expressing canine OS cell lines. In the present study, we demonstrated that 134-mG$_{2a}$-f could recognize endogenous dEGFR by flow cytometric (Fig. 1A, B) and immunocytochemical analyses (Fig. 1C) in D-17 cells. The use of an anti-EGFR mAb for canine OS with high expression of EGFR may also be effective for canine OS cases that have not responded well to treatment in the past.

The most critical aim of the present study was to investigate the antitumor activity of 134-mG$_{2a}$-f for endogenous dEGFR-expressing canine tumor cells. 134-mG$_{2a}$-f demonstrated growth inhibition of endogenous dEGFR-expressing D-17 cells without body weight loss and skin abnormality (Figs. 3 and 4) although the antitumor activity of 134-mG$_{2a}$-f for D-17 is weaker than that for CHO/dEGFR[16] due to the low expression level of dEGFR in D-17 cells (Fig. 1). These results suggest that 134-mG$_{2a}$-f may be useful for an antibody treatment regimen for dEGFR-expressing canine OS. Future investigations are needed to test the antitumor effects of 134-mG$_{2a}$-f against spontaneously develop canine tumors.

Author Disclosure Statement

No competing financial interests exist.

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