MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 42, Number 1, 2023

DOI: 10.1089/mab.2022.0023

Antitumor Activities in Mouse Xenograft Models of Canine Fibroblastic Tumor by Defucosylated Mouse-Dog Chimeric Anti-HER2 Monoclonal Antibody (H77Bf)

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Human epidermal growth factor receptor 2 (HER2) is a cell surface type I transmembrane glycoprotein that is overexpressed on a variety of solid tumors and transduces the oncogenic signaling upon homo- and heterodimerization with HER families. Anti-HER2 monoclonal antibodies (mAbs) including trastuzumab and its antibody-drug conjugate have been shown to improve patients' survival in HER2-positive breast, gastric, and lung cancers. Canine tumors have advantages as naturally occurring tumor models, and share biological and histological characteristics with human tumors. In this study, we generated a defucosylated version of mouse-dog chimeric anti-HER2 mAb (H77Bf) derived from H_2Mab-77 (mouse IgG_1, kappa). H77Bf possesses the high binding affinity (a dissociation constant: 8.7×10^{-10} M) for a dog HER2 (dHER2)-expressing canine fibroblastic tumor cell line (A-72). H77Bf exhibited antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity for A-72 cells. Moreover, intraperitoneal administration of H77Bf significantly suppressed the development of A-72 tumor compared with the control dog IgG in a mouse xenograft model. These results indicate that H77Bf exerts antitumor activities against dHER2-expressing canine cancers, which could provide a valuable information for canine cancer treatment.

Keywords: HER2, mouse-dog chimeric antibody, antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, canine tumor, antitumor activity

Introduction

Human epidermal growth factor receptor 2 (HER2) overexpression is approximately observed in 20%–30% of human breast cancers, which are associated with poor prognosis and higher rates of recurrence.[1] In 1998, trastuzumab was first approved by the U.S. Food and Drug Administration for treatment of HER2-positive breast cancers[2] and later in HER2-positive gastric cancers.[3] Trastuzumab has been the most effective therapy for HER2-positive breast cancer for more than 20 years.[4] Clinically, the efficacy suggests the involvement of immunologic engagement in antibody therapy.[5]

With the increase in lifespan of both humans and dogs, the increased cancer incidence has been observed as well. In canine tumors, the overexpression of dog HER2 (dHER2) has been reported in 32% of canine mammary tumors,[6] 81% of intestinal tumor, 42% of rectal carcinomas, and 28% of cutaneous squamous cell carcinomas[7] in accordance with the American Society of Clinical Oncology and the College of American Pathologists guidelines for HER2 immunostaining.

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In canine tumors, the initiation, disease progression, gene mutations, growth factors, and immune responses are similar to those of human cancers.[8] Doyle et al demonstrated the vaccine-induced dog epidermal growth factor receptor (dEGFR)/dHER2-specific immunity in spontaneous canine cancer.[9] Dogs with EGFR-positive tumors were immunized with a peptide of the dEGFR extracellular domain highly homologous to dHER2. Serum analyses showed high titers of dEGFR/dHER2-binding antibodies with biological activity similar to that of cetuximab and trastuzumab. The antibodies inhibited EGFR intracellular signaling and tumor growth. These results support the approach of antitumor immunity to dEGFR/dHER2 that could be relevant in combination with other immune therapies including immune checkpoint inhibitors.

Previously, we developed anti-HER2 monoclonal antibodies (mAbs), such as H_2Mab-19,[10–13] H_2Mab-41,[14,15] H_2Mab-77 (mouse IgG_1, kappa),[16] H_2Mab-119,[17] H_2Mab-139,[18] and H_2Mab-181,[19,20] which detect not only HER2 but also dHER2. In this study, we produced a defucosylated mouse-dog chimeric anti-HER2 mAb (H77Bf) from H_2Mab-77, and investigated the ability of H77Bf to induce the antitumor efficacy against dHER2-positive tumors.

Materials and Methods

Cell lines

A canine fibroblastic cell line (A-72) was obtained from the American Type Culture Collection (Manassas, VA). A-72 was cultured in Dulbecco's modified Eagle medium (DMEM; 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.). The cell lines were maintained at 37°C in a humidified atmosphere under 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 antitumor activity were approved by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (Permit No. 2021-056). 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 weeks period of each experiment. We determined the loss of original body weight to a point >25% and/or a maximum tumor size >2000 mm^3 as humane endpoints for euthanasia. Mice were euthanized by cervical dislocation; death was verified by respiratory and cardiac arrest.

Antibodies

To generate H77B, we subcloned V_H cDNA of H_2Mab-77 and C_H of dog IgGB into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) along with V_L cDNA of H_2Mab-77 and C_L cDNA of dog kappa light chain into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation), respectively. The vector of H77B was transfected into BINDS-09 cells (FUT8-deficient ExpiCHO-S cells) using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.). The resulting mAb, H77Bf, was purified with Ab-Capcher (ProteNova, Kagawa, Japan). Dog IgG was purchased from Jackson ImmunoResearch, Inc. (West Grove, PA).

Flow cytometric analysis

A-72 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 H77Bf or control blocking buffer for 30 min at 4°C. Then, cells were incubated in the fluorescein isothiocyanate (FITC)-conjugated anti-dog IgG at a dilution of 1:1000 (Thermo Fisher Scientific, Inc.) for 30 min at 4°C. Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Determination of binding affinity of H77Bf to A-72

A-72 cells were suspended in 100 μL of serially diluted H77Bf (0.006–25 μg/mL) followed by the 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).

Antibody-dependent cellular cytotoxicity activity of H77Bf

Canine mononuclear cells (MNCs) were obtained from Yamaguchi University and resuspended in DMEM with 10% FBS to be used as effector cells.[21] Target cells were labeled with 10 μg/mL Calcein AM (Thermo Fisher Scientific, Inc.) and resuspended in the same medium.[22] The target cells (2×10^4 cells/well) were plated in 96-well plates and mixed with effector cells (effector/target cells ratio, 50), 100 μg/mL of H77Bf or control dog IgG. After 4.5 hours 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 after 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 of H77Bf

Cells were labeled with 10 μg/mL Calcein AM and resuspended in the medium.[23] 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 H77Bf or control dog IgG. After 4.5 hours of incubation at 37°C, we measured Calcein release into the supernatant for

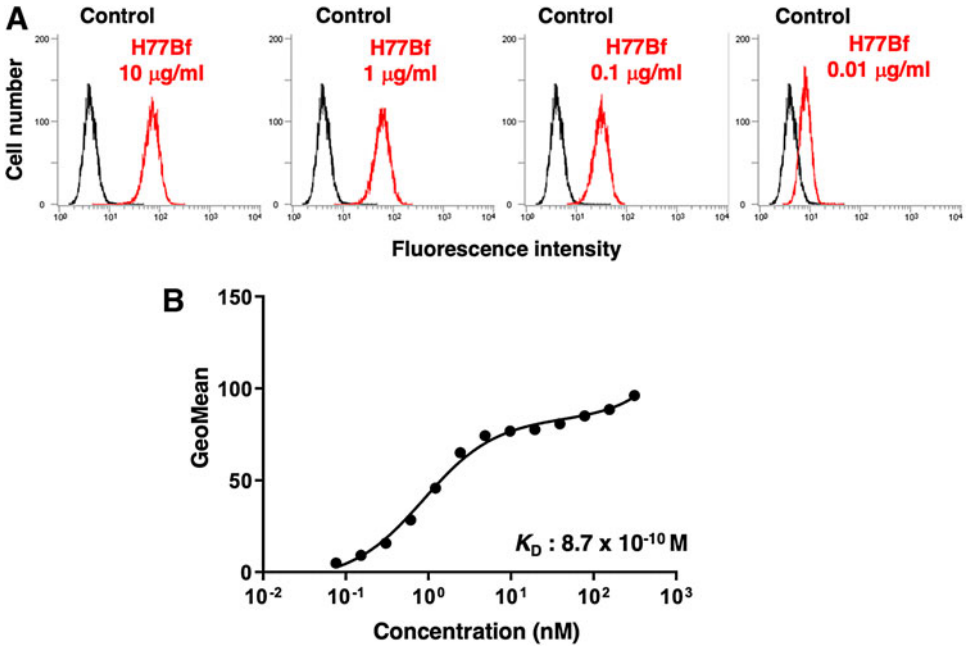

FIG. 1. Flow cytometry using H77Bf. **(A)** A-72 cells were treated with 10, 1, 0.1, and 0.01 μg/mL of H77Bf (red lines) or buffer control (black lines), followed by the treatment with FITC-conjugated anti-dog IgG. **(B)** Kinetic analysis of H77Bf for A-72 cells using flow cytometry. A-72 cells were suspended in 100 μL of H77Bf, followed by the treatment with FITC-conjugated anti-dog IgG. FITC, fluorescein isothiocyanate.

each well. Fluorescence intensity was calculated as described in the Antibody-Dependent Cellular Cytotoxicity Activity (ADCC) of H77Bf section earlier.

Antitumor activity of H77Bf in xenografts of the A-72 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 8 weeks of age. The A-72 cells (0.3 mL of 1.33×10^8 cells/mL in DMEM) were mixed with 0.5 mL 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 8 postinoculation, 100 μg of H77Bf ($n = 8$) or control dog IgG ($n = 8$) in 100 μL PBS were injected intraperitoneally. Additional antibody inoculations were performed on days 14 and 21. MNCs were injected surrounding the tumors on days 8, 14, and 21. The tumor volume was measured on days 7, 10, 14, 17, 21, 24, and 28 after the injection of cells. At day 28 after cell implantation, all mice were euthanized by cervical dislocation. Tumor diameters and volumes were determined as previously described.[24]

Statistical analyses

All data are expressed as mean ± standard error of the mean. Statistical analysis was conducted with Welch's *t*-test for ADCC, complement-dependent cytotoxicity (CDC), and 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 cytometric analysis against the A-72 cells using H77Bf

In previous study, we established an anti-HER2 mAb (H_2Mab-77) using cancer-specific mAb method.[16] In this study, we produced a defucosylated mouse-dog chimeric anti-HER2 mAb (H77Bf) by combining V_H and V_L of H_2Mab-77 with C_H and C_L of dog IgG, respectively. H77Bf reacted with a canine cell line, A-72 cells in a dose-dependent manner (Fig. 1A).

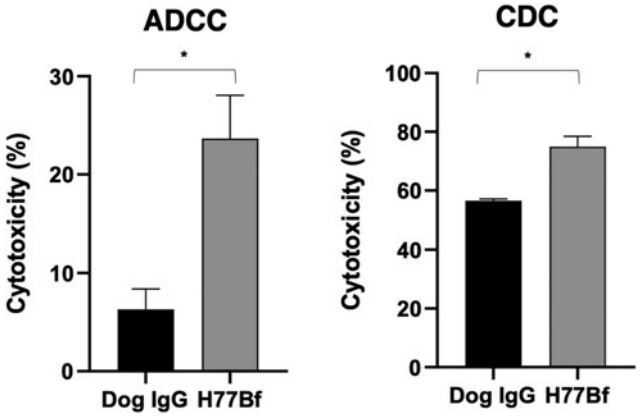

FIG. 2. Evaluation of ADCC and CDC elicited by H77Bf. ADCC (left panel) and CDC (right panel) elicited by H77Bf or control dog IgG targeting A-72 cells were evaluated. The values are expressed as mean ± SEM. Asterisks indicate statistical significance (*$p < 0.05$). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; SEM, standard error of the mean .

Next, we measured the dissociation constant (K_D) between H77Bf and the A-72 cells using flow cytometry. As a result, the K_D was determined to be 8.7×10^{-10} M, indicating that H77Bf exhibits high affinity for A-72 cells (Fig. 1B).

ADCC and CDC activities mediated by H77Bf against the A-72 cells

We next measured the ADCC and CDC activities of H77Bf against the A-72 cells. H77Bf showed ADCC activity against A-72 cells (23.7% cytotoxicity) more effectively compared with the control dog IgG (6.32% cytotoxicity; $p < 0.05$) as shown in Figure 2 (left). H77Bf also showed CDC activity against A-72 cells (75.0% cytotoxicity) more effectively compared with the control dog IgG (56.5% cytotoxicity; $p < 0.05$) as shown in Figure 2 (right). These results indicated that H77Bf mediated significantly higher levels of ADCC and CDC against A-72 cells.

Antitumor activities of H77Bf in the mouse xenografts of A-72 cells

H77Bf (100 μg) or control dog IgG (100 μg) were injected intraperitoneally into mice on days 8, 14, and 21, after the A-72 cell inoculation. The tumor volume was measured on days 7, 10, 14, 17, 21, 24, and 28 after the A-72 cell inoculation. Administration of H77Bf induced the significant reduction of tumor volume on day 10 ($p < 0.05$), 14 ($p < 0.01$), 17 ($p < 0.01$), 21 ($p < 0.01$), 24 ($p < 0.01$), and 28 ($p < 0.01$) compared with that of the control dog IgG administration (Fig. 3A). The administration of H77Bf resulted in a 52.3% tumor volume reduction compared with that of the control dog IgG on day 28 postinoculation.

The A-72 tumors were resected from mice on day 28, and their weights were measured. As a result, the tumor weight of H77Bf-treated mice was significantly less than that of control dog IgG-treated mice (57.8% reduction; $p < 0.01$) (Fig. 3B, C).

Total body weight of the A-72 xenograft mice did not significantly differ among the two groups (Fig. 4A). The body appearance of the A-72 xenograft mice on day 28 is presented in Figure 4B.

Taken together, these results indicate that the administration of H77Bf effectively reduced the growth of A-72 xenograft.

Discussion

Among canine IgG subclasses (A, B, C, and D), the B and D subclasses are involved in ADCC.[25] In this study, we converted mouse IgG_1 into type B of dog IgG, and produced a defucosylated mAb, which has been proved to exhibit more

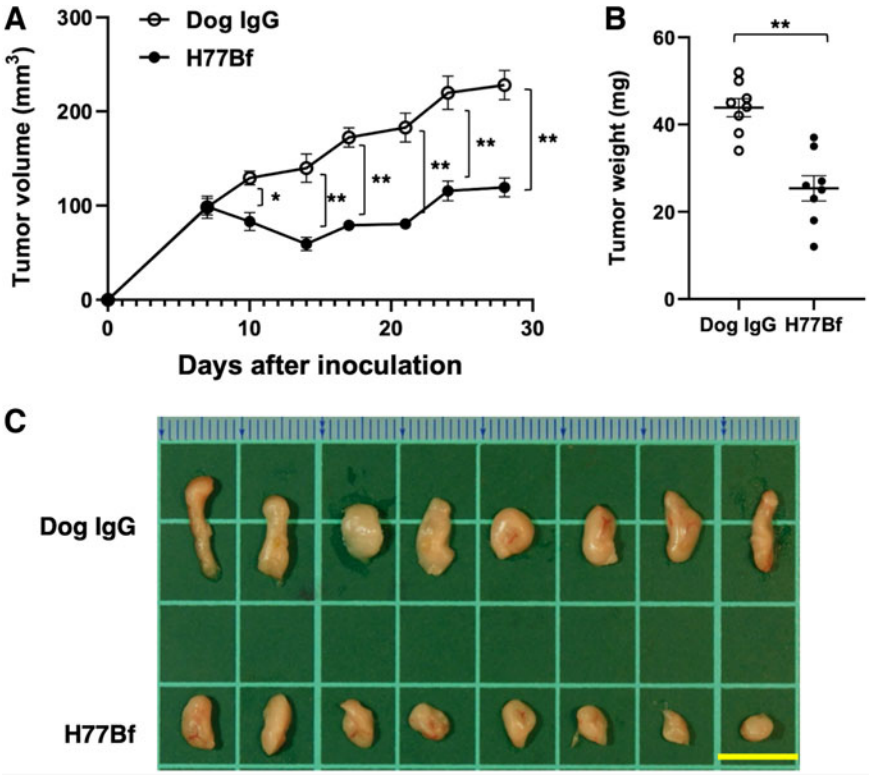

FIG. 3. Evaluation of antitumor activity of H77Bf in A-72 xenograft models. **(A)** Evaluation of tumor volume of A-72 xenograft. A-72 cells (5×10^6 cells) were subcutaneously inoculated into the left flank. On day 8, 100 µg of H77Bf ($n = 8$) or control dog IgG ($n = 8$) in 100 µL PBS was intraperitoneally injected into the mice; additional antibodies were injected on days 14 and 21. The tumor volume was measured on days 7, 10, 14, 17, 21, 24, and 28 after the A-72 cell inoculation. Asterisk indicates statistical significance (**$p < 0.01$, *$p < 0.05$). **(B)** Evaluation of tumor weight in the A-72 xenograft model. The tumor weight on day 28 was measured from the excised A-72 xenograft. The values are expressed as mean ± SEM. Asterisk indicates statistical significance (**$p < 0.01$). **(C)** Appearance of resected tumors of the A-72 xenografts. The tumors of the A-72 xenograft were resected from the H77Bf and control dog IgG groups on day 28. Scale bar, 1 cm. PBS, phosphate-buffered saline.

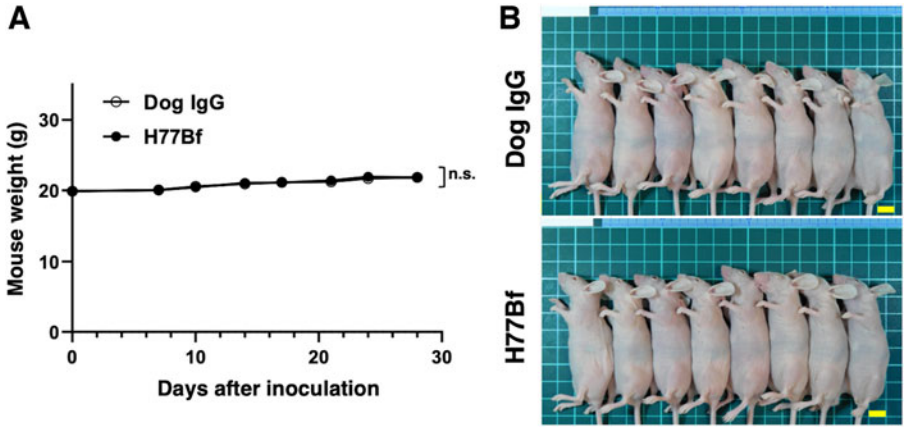

FIG. 4. Body weights and appearance of the mice inoculated with A-72 xenograft. **(A)** Body weights of the mice inoculated with the A-72 xenograft. Body weights of mice inoculated with the A-72 xenograft were recorded on days 7, 10, 14, 17, 21, 24, and 28 after the A-72 cell inoculation (n.s., not significant). **(B)** Body appearance of the mice inoculated with the A-72 xenograft on day 28. Scale bar, 1 cm.

potent ADCC activity through binding to FcγRIIIa on NK cells.[26] We first confirmed the cross-reactivity of H77Bf to A-72 cells (Fig. 1A), and found that H77Bf possesses comparable high binding affinity to A-72 cells (8.7×10^{-10} M, Fig. 1B), compared with human breast cancer SK-BR-3 cells (7.3×10^{-9} M, determined by H_2Mab-77) as reported previously.[16] The quantitative analysis of affinity is important to apply an anti-human molecule mAb to dogs.

The H77Bf administration with canine MNCs resulted in significant growth suppression in A-72 tumor (Fig. 3). This result provided the evidence of H77Bf as a promising antibody therapy against dHER2-positive tumors. We also showed the increased ADCC and CDC activities by H77Bf with canine MNCs and complement, respectively (Fig. 2). These results suggest that ADCC and CDC activity could be involved in the antitumor activity by H77Bf. In the future study, the evaluation of H77Bf against spontaneously developed dHER2-positive canine tumors will be required.

A canine fibroblastic tumor cell line, A-72, was established from a tumor surgically removed from a golden retriever and mainly used for virus research.[27] Although the histology of the original tumor has not been identified, the A-72 cells expressed both dHER2 (Fig. 1) and dEGFR.[22] Furthermore, the cytosolic dot-like staining of dEGFR was observed in the A-72 cells, suggesting that the internalized dEGFR accumulates in the cytoplasm.[22] Therefore, internalization of HER2 by H77B should be evaluated for the development of antibody-drug conjugate. Trastuzumab deruxtecan (T-DXd, DS-8201) is a HER2-targeting antibody conjugated with a novel DNA topoisomerase I inhibitor.[28]

T-DXd showed promising clinical outcomes in metastatic breast cancer.[29] Currently, the clinical efficacy and safety of T-DXd have been evaluated in various tumors. T-DXd has been approved in not only HER2-positive breast cancers,[29–31] but also HER2-mutant lung cancers.[30] We previously generated a mouse-canine chimeric mAb against dog podoplanin[32–34] (P38B) conjugated with emtansine as the payload (P38B-DM1).[35] P38B-DM1 showed cytotoxicity to podoplanin-positive cells and exhibited potent antitumor activity than P38B in the xenograft model.[35]

Immunohistochemistry (IHC) is useful as a diagnostic tool for the identification of neoplasms with histopathology. IHC is routinely used to assist with the diagnosis and to determine the specific treatment (e.g., trastuzumab) for human cancer patients. Although IHC is not routinely used in canine tumors, growing number of studies have been looking for reliable diagnostic and/or prognostic IHC biomarkers including dHER2.[36] Therefore, the standardization of dHER2 IHC is necessary similar to human breast cancer diagnosis. Our established H_2Mab-77 mAb can be used for IHC,[16] and its caninized H77Bf exerts the antitumor activity against dHER2 positive cells (Fig. 3); therefore, the combination of H_2Mab-77 and H77Bf could be applied for both diagnosis and therapy for dHER2-expressed canine tumors.

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 numbers JP22ama121008 (to Y.K.), JP22am0401013 (to Y.K.), JP22bm1004001 (to Y.K.), JP22ck0106730 (to Y.K.), and JP21am0101078 (to Y.K.).

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Received: June 1, 2022
Accepted: September 30, 2022