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Article

An Anti-EpCAM Monoclonal Antibody (EpMab-37-mG2a-f) Exerts Antitumor Activity against Breast Cancer in Mouse Xenograft Model

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Abstract: The epithelial cell adhesion molecule (EpCAM) is a stem cell and carcinoma antigen, which mediates cellular adhesion and proliferative signaling by the proteolytic cleavage. In contrast to low expression in normal epithelium, EpCAM is frequently overexpressed in various carcinomas, which correlates with poor prognosis. Therefore, EpCAM has been considered as a promising target for tumor diagnosis and therapy. Using the Cell-Based Immunization and Screening (CBIS) method, we previously established an anti-EpCAM monoclonal antibody (EpMab-37; mouse IgG_1, kappa). In this study, we investigated the antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and an antitumor activity by a defucosylated mouse IgG_{2a}-type of EpMab-37 (EpMab-37-mG_{2a}-f) against an EpCAM-expressing breast cancer cell line (BT-474). EpMab-37-mG_{2a}-f recognized BT-474 cells with a moderate binding-affinity [a dissociation constant (K_D): 2.9×10^{-8} M] by flow cytometry. EpMab-37-mG_{2a}-f exhibited ADCC and CDC for BT-474 cells by murine splenocytes and complements, respectively. Furthermore, administration of EpMab-37-mG_{2a}-f significantly suppressed the BT-474 xenograft tumor development compared with the control mouse IgG. These results indicated that EpMab-37-mG_{2a}-f exerts antitumor activities against the BT-474 xenograft, and could provide valuable therapeutic regimen for the breast cancers.

Keywords: EpCAM; breast cancer; antitumor activities; antibody-dependent cellular cytotoxicity

1. Introduction

EpCAM is a unique type I transmembrane glycoprotein which is expressed on the basolateral membrane of epithelial cells [1]. EpCAM mediates homophilic and intercellular adhesion through the extracellular domain, which is essential for the epithelial integrity [2]. EpCAM was the first identified human tumor antigen [3], and the expression is correlated with poor prognosis in various tumors [4-7]. EpCAM also functions as a signaling molecule. The formation in intercellular EpCAM oligomers triggers the transmembrane proteolytic cleavage by a membrane protease complex. The EpCAM intracellular

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domain serves as a transcriptional cofactor by interacting with β-catenin, and regulates the transcriptional targets involved in cell proliferation, survival, and stemness [8]. Therefore, overexpression of EpCAM plays critical roles in malignant progression of tumors.

Circulating tumor cells (CTCs) are an important indicator of micro-metastasis, and provide prognostic and therapeutic information [9]. CellSearch® is a platform that captures EpCAM-positive circulating tumor cells (CTCs) from whole blood samples [10]. The U.S. Food and Drug Administration approved CellSearch® in 2009. The EpCAM-based CellSearch® CTC test has been studied in several clinical trials in lung [11], prostate [12], and breast [13] cancers.

The clinicopathological classiffications of breast cancer are based on the expression of estrogen receptor (ER), progesteron receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67. The intrinsic subtypes are classified into luminal A (ER+ and/or PR+, HER2−, and low Ki-67), luminal B HER2-negative (ER+ and/or PR+, HER2−, and high Ki-67), luminal B HER2-positive (ER+ and/or PR+, HER2+), HER2-positive non-luminal (ER− and PR−, HER2+), and triple negative (ER−, PR−, HER2−) [14]. EpCAM expression in breast cancer was previously analyzed and showed varied clinical outcomes in the intrinsic subtypes. Especially, EpCAM expression was associated with an unfavorable prognosis in the luminal B HER2-positive and triple negative subtypes [15].

EpCAM was the first target of monoclonal antibody (mAb) therapy in humans [16]. Adecatumumab is a human recombinant mAb [17], and exhibited antitumor activity in colorectal and prostate cancers through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [18]. Catumaxomab is a bispecific and trifunctional anti-EpCAM/CD3-antibody. Catumaxomab recognizes EpCAM on tumors, recruits T cells through the anti-CD3 arm, and recruits natural killer (NK) cells and macrophages through the Fc domain. These events enhanced ADCC activity [19]. Catumaxomab exhibited promising outcomes in several clinical trials [20-22], and was approved by the European Union for the treatment of patients with malignant ascites in 2009 [23].

We previously established an anti-EpCAM mAb, EpMab-37 (mouse IgG_1, kappa) using the Cell-Based Immunization and Screening (CBIS) method [24] and produced a subclass-switched and a defucosylated EpMab-37 (EpMab-37-mG_{2a}-f) using FUT8-deficient CHO cells (BINDS-09) to potentiate antitumor activities [25]. In this study, we investigated the ability of EpMab-37-mG_{2a}-f to induce ADCC, CDC, and antitumor activities against an EpCAM-expressing breast cancer cell line, BT-474 derived from the luminal B HER2-positive subtype [26].

2. Materials and Methods

2.1. Cell lines

A human breast cancer cell line (BT-474) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). BT-474 was cultured in Dulbecco's Modified Eagle Medium (DMEM; Nacalai Tesque, Inc. (Nacalai), Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc. (Thermo), Waltham, MA), 100 μg/mL streptomycin, 100 units/mL of penicillin, and 0.25 μg/mL amphotericin B (Nacalai). The cell lines were cultured at 37°C in a humidified atmosphere under 5% CO_2.

2.2. Animals

Animal experiments were performed following regulations and guidelines to minimize animal distress and suffering in the laboratory. Animal experiments for antitumor activity of EpMab-37-mG_{2a}-f were approved (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 with food and water supplied ad libitum in a specific pathogen-free environment across the experimental period. Mice were monitored for weight and health every 2-5 days during the experiments.

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The loss of original body weight was determined to a point >25% and/or a maximum tumor size >3,000 mm^3 as humane endpoints for euthanasia.

2.3. Antibodies

An anti-EpCAM mAb, EpMab-37, was established as previously described [27]. To switch the subclass of EpMab-37 from mouse IgG_1 to mouse IgG_{2a} (EpMab-37-mG_{2a}), we subcloned V_H cDNA of EpMab-37 and C_H of mouse IgG_{2a} into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation (Wako), Osaka, Japan). V_L cDNA of EpMab-37 and C_L cDNA of mouse kappa light chain were also subcloned into the pCAG-Neo vector (Wako). To generate the defucosylated EpMab-37-mG_{2a}, the vector for the EpMab-37-mG_{2a} was transduced into BINDS-09 (FUT8 knockout ExpiCHO-S) cells using the ExpiCHO Expression System (Thermo) [28-42]. Defucosytaled EpMab-37-mG_{2a} (EpMab-37-mG_{2a}-f) was purified using Ab-Capcher (ProteNova Co., Ltd., Kanagawa, Japan). Mouse IgG (cat. no. 140-09511) and IgG_{2a} (cat. no. M7769) were purchased from Wako and Sigma-Aldrich (St. Louis, MO), respectively.

2.4. Flow cytometry

BT-474 cells were harvested by 0.25% trypsin/1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai). After washing with blocking buffer: phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA; Nacalai), cells were treated with EpMab-37-mG_{2a}-f or blocking buffer as a negative control for 30 min at 4°C. Then, cells were incubated in Alexa Fluor 488-conjugated anti-mouse IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA) for 30 min at 4°C. Fluorescence data were collected by the SA3800 Cell Analyzer and analyzed by SA3800 software ver. 2.05 (Sony Corp., Tokyo, Japan).

2.5. Determination of binding affinity

Serially diluted EpMab-37-mG_{2a}-f (0.006-100 μg/mL) was suspended with BT-474 cells. The cells were further treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:200). Fluorescence data were obtained by BD FACSLyric (BD Biosciences, San Jose, CA). To determine the dissociation constant (K_D), the fitting binding isotherms to built-in one-site binding models in GraphPad Prism 9 (GraphPad Software, La Jolla, CA) was used.

2.6. ADCC

To assess the ADCC induction by EpMab-37-mG_{2a}-f, four female five-week-old BALB/c nude mice (mean weight, 15 ± 3 g) were purchased from Charles River Laboratories, Inc. (Kanagawa, Japan). After euthanasia by cervical dislocation, spleens were removed aseptically, and single-cell suspensions were obtained by forcing spleen tissues through a sterile cell strainer (product no. 352360; BD Falcon; Corning, Inc., New York, NY) with a syringe.

Erythrocytes were lysed with a 10-sec exposure to ice-cold distilled water. The splenocytes were washed with DMEM and resuspended in DMEM with 10% FBS; this preparation was designated as effector cells. BT-474 cells were labeled with 10 μg/mL Calcein-AM (Thermo) and resuspended in the same medium. BT-474 cells were then transferred to 96-well plates, at 2×10^4 cells/well, and mixed with effector cells at an effector-to-target ratio of 100:1, along with 100 μg/mL of EpMab-37-mG_{2a}-f or control mouse IgG_{2a}. After a 4.5-h incubation at 37°C, Calcein release into the supernatant was measured for each well. Fluorescence intensity was assessed 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 was measured as a percentage of lysis and calculated using the equation: Percentage of lysis (%) = (E − S) / (M − S) × 100, where E is the fluorescence measured in combined cultures of BT-474 and effector cells, S is the spontaneous fluorescence of the target cells, and M is the maximum fluorescence measured after lysis

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of all cells with buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4), and 10 mM EDTA.

2.7. CDC

BT-474 cells were labeled with 10 μg/mL Calcein-AM, resuspended in medium and plated in 96-well plates, at 2 × 10^4 cells/well, with 10% rabbit complement (Low-Tox-M rabbit complement; Cedarlane Laboratories, Hornby, Ontario, Canada), 100 μg/mL of EpMab-37-mG_{2a}-f, or control mouse IgG_{2a} added to each well. After 4.5 h of incubation at 37°C, Calcein release into the supernatant was measured for each well. Fluorescence intensity was calculated as described in the ADCC section above.

2.8. Antitumor activities in xenografts of BT-474

BALB/c nude mice (female, 4 weeks old) were purchased from Charles River Laboratories, Inc. BT-474 cells (5x10^6 cells) were resuspended in DMEM and mixed with BD Matrigel Matrix Growth Factor Reduced (BD Biosciences) were subcutaneously injected into the left flank of 10 weeks old mice. On day 6 post-inoculation, 100 μg of EpMab-37-mG_{2a}-f (n=8) or control mouse IgG (n=8) in 100 μl PBS were intraperitoneally injected. On day 13 and 18, additional antibody inoculations were performed. The tumor volume was measured on days 6, 11, 13, 18, 22 and 24 after the injection of cells. Tumor volumes were determined as previously described [28-46].

2.9. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was conducted with Welch's *t* test for ADCC activity, CDC activity, and tumor weight. ANOVA with Sidak's post hoc test were conducted for tumor volume and mouse weight. GraphPad Prism 8 (GraphPad Software, Inc.) was used for all calculations. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Flow cytometric analysis

In our previous study, a sensitive and specific anti-EpCAM mAb, EpMab-37, was established using the Cell-Based Immunization and Screening (CBIS) method [27]. We first performed flow cytometric analysis using EpMab-37-mG_{2a}-f against BT-474 cells, and found that EpMab-37-mG_{2a}-f recognized the BT-474 cells (Fig. 1A). A kinetic analysis of the interactions of EpMab-37-mG_{2a}-f with BT-474 cells was conducted using flow cytometry. The K_D of EpMab-37-mG_{2a}-f to BT-474 cells were 2.9 × 10^{-8} M (Fig. 1B), indicating moderate binding affinity of EpMab-37-mG_{2a}-f against BT-474 cells.

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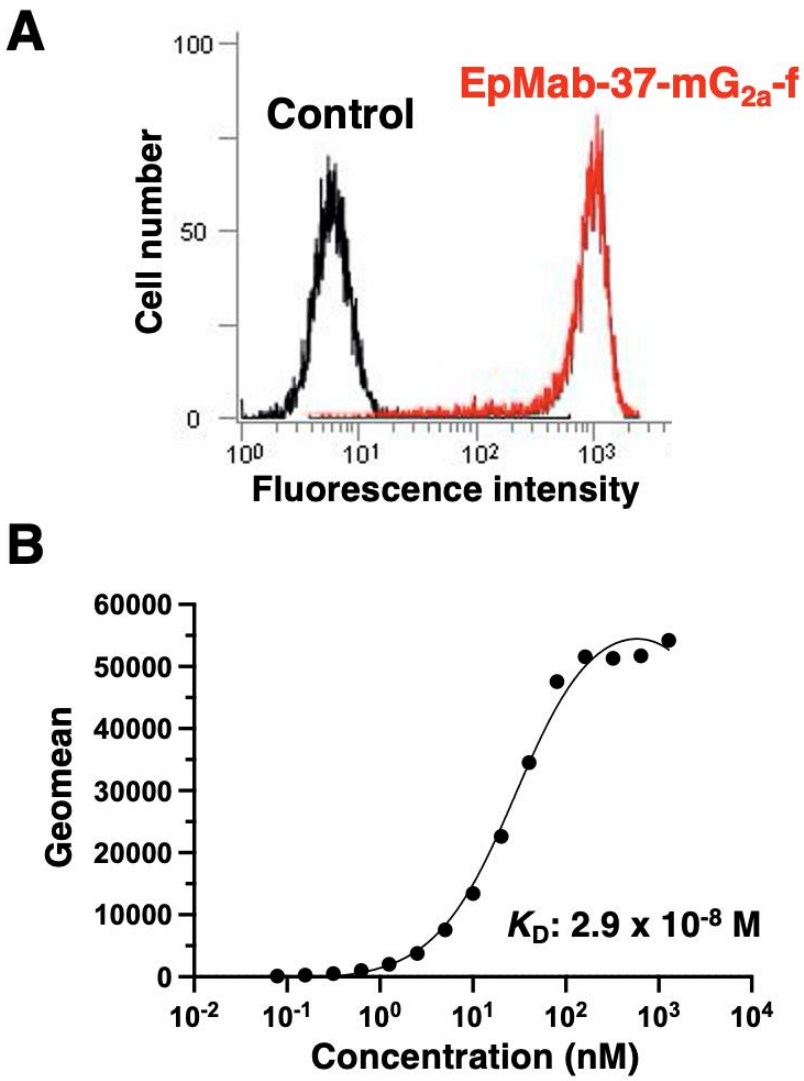

Figure 1. Flow cytometric analyses. (A) BT-474 cells were treated with EpMab-37-mG2a-f (red line) or blocking buffer as a negative control (black line), followed by addition of Alexa Fluor 488-conjugated anti-mouse IgG. Data were collected using the EC800 Cell Analyzer. (B) To determine binding affinity of EpMab-37-mG2a-f for BT-474 cells, BT-474 cells were suspended in 100 μL of serially diluted EpMab-37-mG2a-f (0.006-100 μg/mL), followed by the addition of Alexa Fluor 488-conjugated anti-mouse IgG. Data were collected using the BD FACS Lyric and analyzed by GraphPad PRISM 9. *K*D, dissociation constant.

3.2. ADCC and CDC activities of EpMab-37-mG2a-f in a breast cancer cell line (BT-474)

We next examined whether EpMab-37-mG2a-f induces ADCC and CDC activities in an EpCAM-expressing BT-474 cells. EpMab-37-mG2a-f exhibited much higher ADCC (64.0% cytotoxicity) in BT-474 cells than that of control mouse IgG2a (8.49% cytotoxicity; $P < 0.01$) (Fig. 2A). EpMab-37-mG2a-f also showed higher CDC activity (64.2% cytotoxicity) in BT-474 cells than that of control mouse IgG2a (11.1% cytotoxicity; $P < 0.01$) (Fig. 2B). These results demonstrated that EpMab-37-mG2a-f exhibited potent ADCC and CDC activities against BT-474 cells.

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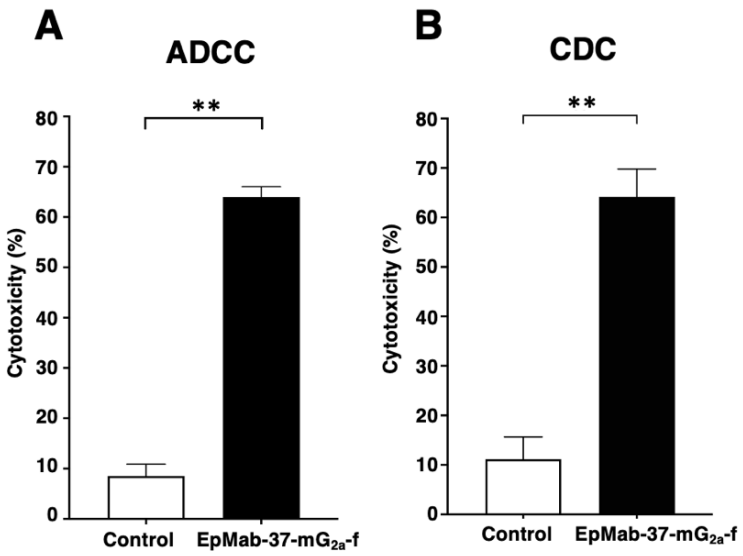

Figure 2. Evaluation of EpMab-37-mG$_{2a}$-f mediated ADCC and CDC activities in BT-474 cells. (A) ADCC elicited by EpMab-37-mG$_{2a}$-f or control mouse IgG$_{2a}$ against BT-474 cells. (B) CDC elicited by EpMab-37-mG$_{2a}$-f or control mouse IgG$_{2a}$ against BT-474 cells. Values are shown as mean ± SEM. Asterisks indicate statistical significance (**P < 0.01; Welch's *t*-test). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity.

3.3. Antitumor effects of EpMab-37-mG$_{2a}$-f in a mouse xenograft model of BT-474

BT-474 cells were inoculated into the left flank of mice, followed by the intraperitoneally injection of EpMab-37-mG$_{2a}$-f or control mouse IgG on days 6, 13, and 18. The tumor volume was measured on days 6, 11, 13, 18, 22, and 24 after the BT-474 cell inoculation. EpMab-37-mG$_{2a}$-f-treated mice exhibited significantly less tumor volume on day 11 ($P < 0.01$), day 13 ($P < 0.01$), day 18 ($P < 0.01$), day 22 ($P < 0.01$), and day 24 ($P < 0.01$), compared with control mouse IgG-treated control mice (Fig. 3). The EpMab-37-mG$_{2a}$-f treatment resulted in a 51.3% reduction of the tumor volume compared with that of the control mouse IgG on day 24 (Fig. 3). Tumors from EpMab-37-mG$_{2a}$-f-treated mice weighed significantly less than tumors from control IgG-treated control mice (52.4% reduction, $P < 0.01$; Fig. 4A). Resected tumors on day 24 are presented in Fig. 4B. The total body weights did not significantly differ between the EpMab-37-mG$_{2a}$-f-treatment and the control groups (Fig. 5A). The body appearance of mice on day 24 post inoculation is shown in Fig. 5B, and the body weights loss and skin disorder were not observed. These results indicated that administration of EpMab-37-mG$_{2a}$-f significantly reduced the growth of BT-474 xenografts.

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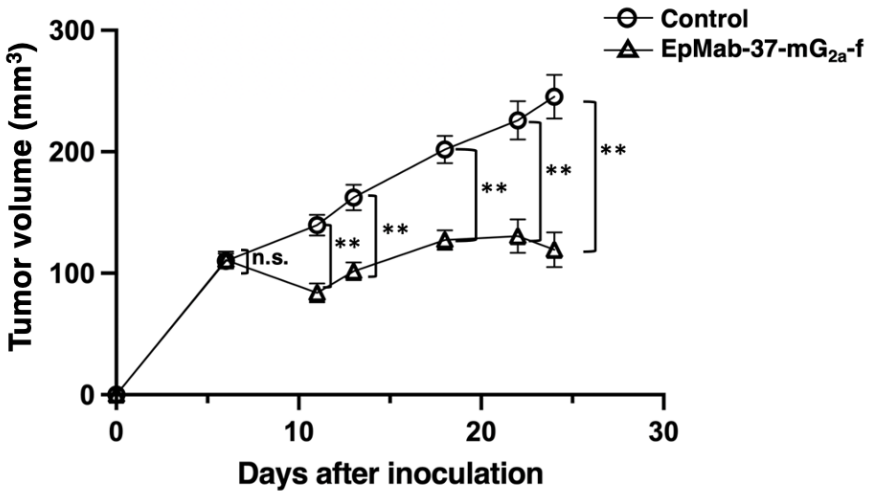

Figure 3. Evaluation of tumor volume in BT-474 xenograft. BT-474 cells (5×10^6 cells) were injected subcutaneously into the left flank of mice. Mice received intraperitoneal injections of 100 μg of EpMab-37-mG$_{2a}$-f or control mouse IgG in 100 μL PBS on days 6, 13, and 18 post tumor inoculation. The tumor volume was measured on days 6, 11, 13, 18, 22 and 24 post tumor inoculation. Values are shown as means ± SEM. Asterisks indicate statistical significance (**P < 0.01; n.s., not significant; ANOVA and Sidak's multiple comparisons test).

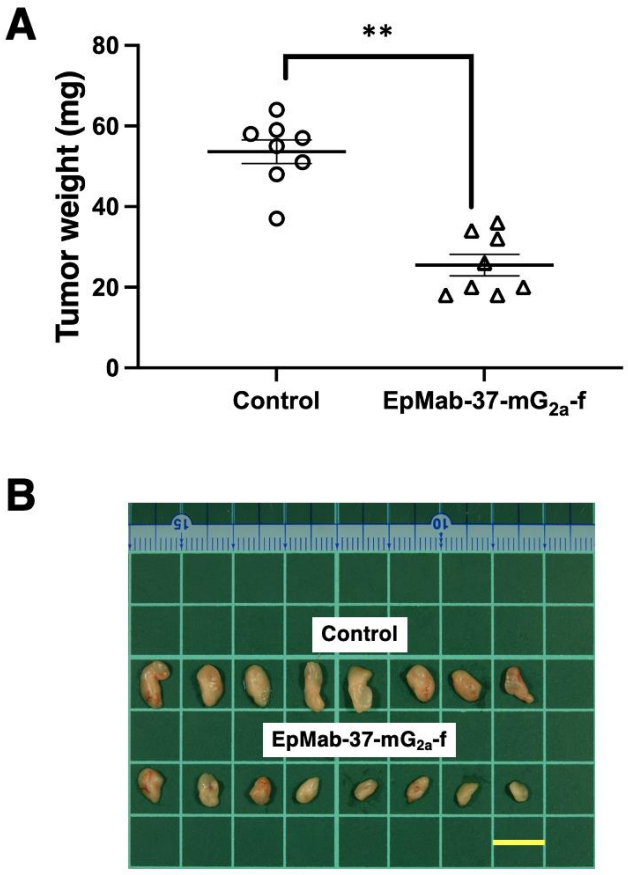

Figure 4. Evaluation of tumor weight in BT-474 xenograft model. (A) On day 24 post inoculation, tumors were resected from EpMab-37-mG$_{2a}$-f and control mouse IgG groups, and tumors were weighed. Values are shown as mean ± SEM. Asterisk indicates statistical significance (**P < 0.01, Welch's *t*-test). (B) Images for tumors obtained from BT-474 mice xenograft belonging to EpMab-37-mG$_{2a}$-f and control mouse IgG groups, resected on day 24. Scale bar, 1 cm.

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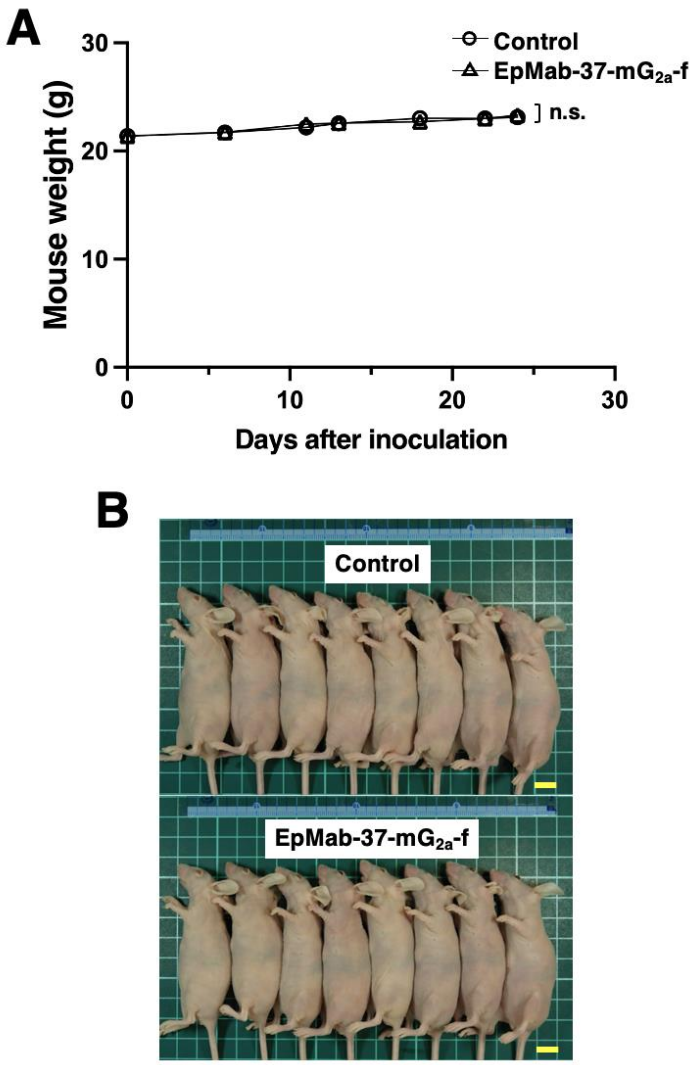

Figure 5. Body weights and appearance of the mice. (A) Body weights of the mice implanted with BT-474 xenografts were recorded on days 6, 11, 13, 18, 22 and 24 (n.s.: not significant; ANOVA and Sidak's multiple comparisons test). (B) Body appearance of the mice on day 24. Scale bar, 1 cm.

4. Discussion

The impact of EpCAM expression on breast cancer prognosis is dependent on intrinsic subtype. In the luminal B HER2-positive and triple negative subtypes, EpCAM expression is associated with an unfavorable prognosis. In contrast, EpCAM expression is associated with a favorable prognosis in the HER2-positive non-luminal subtype [15]. Therefore, the luminal B HER2-positive and triple negative subtypes are potential groups for treatment with EpCAM-targeting therapy. In this study, we investigated the antitumor effect of a defucosylated anti-EpCAM mAb (EpMab-37-mG$_{2a}$-f) against a breast cancer cell line, BT-474 derived from luminal B HER2-positive subtype [26]. EpMab-37-mG$_{2a}$-f exhibited superior ADCC and CDC activities *in vitro* (Fig. 2), and antitumor activity against BT-474 xenograft in nude mice (Fig. 3 and 4). We previously developed an anti-HER2 mAb (H$_2$Mab-19) and examined ADCC, CDC, and antitumor activities against BT-474 cells [38]. Although the binding affinity of H$_2$Mab-19 and EpMab-37-mG$_{2a}$-f to BT-474 cells were comparable, EpMab-37-mG$_{2a}$-f exerted more potent ADCC activity and antitumor effect *in vivo*. These results are probably due to the defucosylation in EpMab-37-mG$_{2a}$-f, but not in H$_2$Mab-19. Moreover, EpCAM forms a cis-dimer which further makes a biologically relevant oligomeric state (e.g. cis-tetramer, trans-tetramer, and trans-octamer) according to several experimental observations [47]. These oligomeric structure of EpCAM could

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promote the clustering of anti-EpCAM mAbs, which might help the FcγRIIIa engagement on effector cells, and potentiate the ADCC activity.

EpCAM is an important cell surface molecule to collect CTCs [10]. Recently, CTC expansion techniques have been developed to evaluate of the characteristics of CTCs. The techniques include the two-dimensional (2D) long-term expansion, 3D organoids/spheroids culture, and *in vivo* xenografts/metastasis formation in immunodeficient mice [48]. It would be worthwhile to investigate the effect of EpMab-37-mG$_{2a}$-f on the 2D and 3D CTC expansion *in vitro* and anti-metastatic activity *in vivo.*

We have been investigating the critical epitope of EpMab-37 [24], and recently identified that Arg163 of EpCAM is the most important residue of the EpMab-37 epitope (submitted). Among clinically tested mAbs, no mAb recognized above region, suggesting that EpMab-37 possesses a unique epitope and a different mode of actions. In this study, we did not examine the EpCAM-internalizing activity by EpMab-37-mG$_{2a}$-f. Furthermore, the relationship between the internalizing activity and the epitope has not been investigated. In future study, we would like to evaluate it for the development of antibody-drug conjugates.

Anti-EpCAM mAb can be used for a bispecific Ab with anti-MET mAb [49]. MM-131 is a bispecific Ab that is monovalent against MET, but exhibits high avidity to EpCAM through binding to single chain Fv of an anti-EpCAM mAb, MOC31 [50]. MM-131 exhibits antagonistic activity that interferes both ligand-dependent and ligand-independent MET signaling, and induces the receptor down-regulation [49]. MCLA-128 is a bispecific Ab for HER2 and HER3. MCLA-128 can inhibit heregulin (a HER3 ligand)-mediated signaling of HER2/HER3 heterodimer, and suppress tumor cell growth via the suppression of PI3K/Akt signaling [51]. Clinical studies on MCLA-128 are ongoing in patients with breast cancer, pancreatic cancer, and non-small cell lung cancers [52]. In the future study, we would like to apply EpMab-37 for the combination therapy with anti-HER2 mAbs or develop a bispecific mAb targeting EpCAM and HER2.

Author Contributions: TA, TO, GL, and TY performed the experiments. MKK, MK, and YK designed the experiments. TA, TO, HS, and YK analyzed the data. TA, HS, and YK wrote the manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

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Institutional Review Board Statement: The animal experiments for antitumor activity were performed (approval no. 2022-024) by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (Numazu, Japan).

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Conflicts of Interest: The authors declare no conflict of interest involving this article.

References

1. Trzpis, M.; McLaughlin, P.M.; de Leij, L.M.; Harmsen, M.C. Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. *Am J Pathol* **2007**, *171*, 386-395, doi:10.2353/ajpath.2007.070152.
2. Brown, T.C.; Sankpal, N.V.; Gillanders, W.E. Functional Implications of the Dynamic Regulation of EpCAM during Epithelial-to-Mesenchymal Transition. *Biomolecules* **2021**, *11*, 956, doi:10.3390/biom11070956.
3. Herlyn, M.; Steplewski, Z.; Herlyn, D.; Koprowski, H. Colorectal carcinoma-specific antigen: detection by means of monoclonal antibodies. *Proc Natl Acad Sci U S A* **1979**, *76*, 1438-1442, doi:10.1073/pnas.76.3.1438.

4. Carpenter, G.; Red Brewer, M. EpCAM: another surface-to-nucleus missile. *Cancer Cell* **2009**, *15*, 165-166, doi:10.1016/j.ccr.2009.02.005.
5. Fong, D.; Steurer, M.; Obrist, P.; Barbieri, V.; Margreiter, R.; Amberger, A.; Laimer, K.; Gastl, G.; Tzankov, A.; Spizzo, G. Ep-CAM expression in pancreatic and ampullary carcinomas: frequency and prognostic relevance. *J Clin Pathol* **2008**, *61*, 31-35, doi:10.1136/jcp.2006.037333.
6. Spizzo, G.; Obrist, P.; Ensinger, C.; Theurl, I.; Dünser, M.; Ramoni, A.; Gunsilius, E.; Eibl, G.; Mikuz, G.; Gastl, G. Prognostic significance of Ep-CAM AND Her-2/neu overexpression in invasive breast cancer. *Int J Cancer* **2002**, *98*, 883-888, doi:10.1002/ijc.10270.
7. Varga, M.; Obrist, P.; Schneeberger, S.; Mühlmann, G.; Felgel-Farnholz, C.; Fong, D.; Zitt, M.; Brunhuber, T.; Schäfer, G.; Gastl, G.; et al. Overexpression of epithelial cell adhesion molecule antigen in gallbladder carcinoma is an independent marker for poor survival. *Clin Cancer Res* **2004**, *10*, 3131-3136, doi:10.1158/1078-0432.ccr-03-0528.
8. Eyvazi, S.; Farajnia, S.; Dastmalchi, S.; Kanipour, F.; Zarredar, H.; Bandehpour, M. Antibody Based EpCAM Targeted Therapy of Cancer, Review and Update. *Curr Cancer Drug Targets* **2018**, *18*, 857-868, doi:10.2174/1568009618666180102102311.
9. Xiao, J.; Pohlmann, P.R.; Isaacs, C.; Weinberg, B.A.; He, A.R.; Schlegel, R.; Agarwal, S. Circulating Tumor Cells: Technologies and Their Clinical Potential in Cancer Metastasis. *Biomedicines* **2021**, *9*, doi:10.3390/biomedicines9091111.
10. de Bono, J.S.; Scher, H.I.; Montgomery, R.B.; Parker, C.; Miller, M.C.; Tissing, H.; Doyle, G.V.; Terstappen, L.W.; Pienta, K.J.; Raghavan, D. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res* **2008**, *14*, 6302-6309, doi:10.1158/1078-0432.Ccr-08-0872.
11. Watanabe, M.; Kenmotsu, H.; Ko, R.; Wakuda, K.; Ono, A.; Imai, H.; Taira, T.; Naito, T.; Murakami, H.; Abe, M.; et al. Isolation and molecular analysis of circulating tumor cells from lung cancer patients using a microfluidic chip type cell sorter. *Cancer Sci* **2018**, *109*, 2539-2548, doi:10.1111/cas.13692.
12. Zapatero, A.; Gómez-Caamaño, A.; Cabeza Rodriguez, M.; Muinelo-Romay, L.; Martin de Vidales, C.; Abalo, A.; Calvo Crespo, P.; Leon Mateos, L.; Olivier, C.; Vega Piris, L.V. Detection and dynamics of circulating tumor cells in patients with high-risk prostate cancer treated with radiotherapy and hormones: a prospective phase II study. *Radiat Oncol* **2020**, *15*, 137, doi:10.1186/s13014-020-01577-5.
13. Lampignano, R.; Yang, L.; Neumann, M.H.D.; Franken, A.; Fehm, T.; Niederacher, D.; Neubauer, H. A Novel Workflow to Enrich and Isolate Patient-Matched EpCAM(high) and EpCAM(low/negative) CTCs Enables the Comparative Characterization of the PIK3CA Status in Metastatic Breast Cancer. *Int J Mol Sci* **2017**, *18*, 1885, doi:10.3390/ijms18091885.
14. Goldhirsch, A.; Wood, W.C.; Coates, A.S.; Gelber, R.D.; Thürlimann, B.; Senn, H.J. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Ann Oncol* **2011**, *22*, 1736-1747, doi:10.1093/annonc/mdr304.
15. Soysal, S.D.; Muenst, S.; Barbie, T.; Fleming, T.; Gao, F.; Spizzo, G.; Oertli, D.; Viehl, C.T.; Obermann, E.C.; Gillanders, W.E. EpCAM expression varies significantly and is differentially associated with prognosis in the luminal B HER2(+), basal-like, and HER2 intrinsic subtypes of breast cancer. *Br J Cancer* **2013**, *108*, 1480-1487, doi:10.1038/bjc.2013.80.
16. Sears, H.F.; Atkinson, B.; Mattis, J.; Ernst, C.; Herlyn, D.; Steplewski, Z.; Häyry, P.; Koprowski, H. Phase-I clinical trial of monoclonal antibody in treatment of gastrointestinal tumours. *Lancet* **1982**, *1*, 762-765, doi:10.1016/s0140-6736(82)91811-6.
17. Kurtz, J.E.; Dufour, P. Adecatumumab: an anti-EpCAM monoclonal antibody, from the bench to the bedside. *Expert Opin Biol Ther* **2010**, *10*, 951-958, doi:10.1517/14712598.2010.482098.
18. Naundorf, S.; Preithner, S.; Mayer, P.; Lippold, S.; Wolf, A.; Hanakam, F.; Fichtner, I.; Kufer, P.; Raum, T.; Riethmüller, G.; et al. In vitro and in vivo activity of MT201, a fully human monoclonal antibody for pancarcinoma treatment. *Int J Cancer* **2002**, *100*, 101-110, doi:10.1002/ijc.10443.
19. Schönberger, S.; Kraft, D.; Nettersheim, D.; Schorle, H.; Casati, A.; Craveiro, R.B.; Mohseni, M.M.; Calaminus, G.; Dilloo, D. Targeting EpCAM by a Bispecific Trifunctional Antibody Exerts Profound Cytotoxic Efficacy in Germ Cell Tumor Cell Lines. *Cancers (Basel)* **2020**, *12*, 1279, doi:10.3390/cancers12051279.

20. Ruf, P.; Kluge, M.; Jäger, M.; Burges, A.; Volovat, C.; Heiss, M.M.; Hess, J.; Wimberger, P.; Brandt, B.; Lindhofer, H. Pharmacokinetics, immunogenicity and bioactivity of the therapeutic antibody catumaxomab intraperitoneally administered to cancer patients. *Br J Clin Pharmacol* **2010**, *69*, 617-625, doi:10.1111/j.1365-2125.2010.03635.x.
21. Seeber, A.; Martowicz, A.; Spizzo, G.; Buratti, T.; Obrist, P.; Fong, D.; Gastl, G.; Untergasser, G. Soluble EpCAM levels in ascites correlate with positive cytology and neutralize catumaxomab activity in vitro. *BMC Cancer* **2015**, *15*, 372, doi:10.1186/s12885-015-1371-1.
22. Knödler, M.; Körfer, J.; Kunzmann, V.; Trojan, J.; Daum, S.; Schenk, M.; Kullmann, F.; Schroll, S.; Behringer, D.; Stahl, M.; et al. Randomised phase II trial to investigate catumaxomab (anti-EpCAM × anti-CD3) for treatment of peritoneal carcinomatosis in patients with gastric cancer. *Br J Cancer* **2018**, *119*, 296-302, doi:10.1038/s41416-018-0150-6.
23. Linke, R.; Klein, A.; Seimetz, D. Catumaxomab: clinical development and future directions. *MAbs* **2010**, *2*, 129-136, doi:10.4161/mabs.2.2.11221.
24. Li, G.; Suzuki, H.; Asano, T.; Tanaka, T.; Suzuki, H.; Kaneko, M.K.; Kato, Y. Development of a Novel Anti-EpCAM Monoclonal Antibody for Various Applications. *Antibodies (Basel)* **2022**, *11*, 41, doi:10.3390/antib11020041.
25. Shinkawa, T.; Nakamura, K.; Yamane, N.; Shoji-Hosaka, E.; Kanda, Y.; Sakurada, M.; Uchida, K.; Anazawa, H.; Satoh, M.; Yamasaki, M.; et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem* **2003**, *278*, 3466-3473, doi:10.1074/jbc.M210665200.
26. Subik, K.; Lee, J.F.; Baxter, L.; Strzepek, T.; Costello, D.; Crowley, P.; Xing, L.; Hung, M.C.; Bonfiglio, T.; Hicks, D.G.; et al. The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines. *Breast Cancer (Auckl)* **2010**, *4*, 35-41.
27. Li, G.; Suzuki, H.; Asano, T.; Tanaka, T.; Suzuki, H.; Kaneko, M.K.; Kato, Y. Development of a Novel Anti-EpCAM Monoclonal Antibody for Various Applications. *Antibodies (Basel)* **2022**, *11*, doi:10.3390/antib11020041.
28. Goto, N.; Suzuki, H.; Ohishi, T.; Harakawa, A.; Li, G.; Saito, M.; Takei, J.; Tanaka, T.; Asano, T.; Sano, M.; et al. Antitumor Activities in Mouse Xenograft Models of Canine Fibroblastic Tumor by Defucosylated Anti-Epidermal Growth Factor Receptor Monoclonal Antibody. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 67-73, doi:10.1089/mab.2021.0059.
29. Hosono, H.; Takei, J.; Ohishi, T.; Sano, M.; Asano, T.; Sayama, Y.; Nakamura, T.; Yanaka, M.; Kawada, M.; Harada, H.; et al. Anti-EGFR monoclonal antibody 134-mG2a exerts antitumor effects in mouse xenograft models of oral squamous cell carcinoma. *Int J Mol Med* **2020**, *46*, 1443-1452, doi:10.3892/ijmm.2020.4700.
30. Itai, S.; Ohishi, T.; Kaneko, M.K.; Yamada, S.; Abe, S.; Nakamura, T.; Yanaka, M.; Chang, Y.W.; Ohba, S.I.; Nishioka, Y.; et al. Anti-podocalyxin antibody exerts antitumor effects via antibody-dependent cellular cytotoxicity in mouse xenograft models of oral squamous cell carcinoma. *Oncotarget* **2018**, *9*, 22480-22497, doi:10.18632/oncotarget.25132.
31. Kaneko, M.K.; Ohishi, T.; Nakamura, T.; Inoue, H.; Takei, J.; Sano, M.; Asano, T.; Sayama, Y.; Hosono, H.; Suzuki, H.; et al. Development of Core-Fucose-Deficient Humanized and Chimeric Anti-Human Podoplanin Antibodies. *Monoclon Antib Immunodiagn Immunother* **2020**, *39*, 167-174, doi:10.1089/mab.2020.0019.
32. Kato, Y.; Ohishi, T.; Takei, J.; Nakamura, T.; Kawada, M.; Kaneko, M.K. An Antihuman Epidermal Growth Factor Receptor 2 Monoclonal Antibody (H(2)Mab-19) Exerts Antitumor Activity in Glioblastoma Xenograft Models. *Monoclon Antib Immunodiagn Immunother* **2020**, *39*, 135-139, doi:10.1089/mab.2020.0013.
33. Li, G.; Ohishi, T.; Kaneko, M.K.; Takei, J.; Mizuno, T.; Kawada, M.; Saito, M.; Suzuki, H.; Kato, Y. Defucosylated Mouse-Dog Chimeric Anti-EGFR Antibody Exerts Antitumor Activities in Mouse Xenograft Models of Canine Tumors. *Cells* **2021**, *10*, 3599, doi:10.3390/cells10123599.
34. Nanamiya, R.; Takei, J.; Ohishi, T.; Asano, T.; Tanaka, T.; Sano, M.; Nakamura, T.; Yanaka, M.; Handa, S.; Tateyama, N.; et al. Defucosylated Anti-Epidermal Growth Factor Receptor Monoclonal Antibody (134-mG(2a)-f) Exerts Antitumor Activities in Mouse Xenograft Models of Canine Osteosarcoma. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 1-7, doi:10.1089/mab.2021.0036.

35. Ohishi, T.; Kato, Y.; Kaneko, M.K.; Ohba, S.I.; Inoue, H.; Harakawa, A.; Kawada, M. Anti-Metastatic Activity of an Anti-EGFR Monoclonal Antibody against Metastatic Colorectal Cancer with KRAS p.G13D Mutation. *Int J Mol Sci* **2020**, *21*, doi:10.3390/ijms21176037.
36. Suzuki, H.; Ohishi, T.; Asano, T.; Tanaka, T.; Saito, M.; Mizuno, T.; Yoshikawa, T.; Kawada, M.; Kaneko, M.K.; Kato, Y. Defucosylated mouse-dog chimeric anti-HER2 monoclonal antibody exerts antitumor activities in mouse xenograft models of canine tumors. *Oncol Rep* **2022**, *48*, doi:10.3892/or.2022.8366.
37. Takei, J.; Kaneko, M.K.; Ohishi, T.; Hosono, H.; Nakamura, T.; Yanaka, M.; Sano, M.; Asano, T.; Sayama, Y.; Kawada, M.; et al. A defucosylated anti-CD44 monoclonal antibody 5-mG2a-f exerts antitumor effects in mouse xenograft models of oral squamous cell carcinoma. *Oncol Rep* **2020**, *44*, 1949-1960, doi:10.3892/or.2020.7735.
38. Takei, J.; Kaneko, M.K.; Ohishi, T.; Kawada, M.; Harada, H.; Kato, Y. H(2)Mab-19, an anti-human epidermal growth factor receptor 2 monoclonal antibody exerts antitumor activity in mouse oral cancer xenografts. *Exp Ther Med* **2020**, *20*, 846-853, doi:10.3892/etm.2020.8765.
39. Takei, J.; Ohishi, T.; Kaneko, M.K.; Harada, H.; Kawada, M.; Kato, Y. A defucosylated anti-PD-L1 monoclonal antibody 13-mG(2a)-f exerts antitumor effects in mouse xenograft models of oral squamous cell carcinoma. *Biochem Biophys Rep* **2020**, *24*, 100801, doi:10.1016/j.bbrep.2020.100801.
40. Tanaka, T.; Ohishi, T.; Saito, M.; Suzuki, H.; Kaneko, M.K.; Kawada, M.; Kato, Y. Defucosylated Anti-Epidermal Growth Factor Receptor Monoclonal Antibody Exerted Antitumor Activities in Mouse Xenograft Models of Canine Mammary Gland Tumor. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 142-149, doi:10.1089/mab.2022.0009.
41. Tateyama, N.; Nanamiya, R.; Ohishi, T.; Takei, J.; Nakamura, T.; Yanaka, M.; Hosono, H.; Saito, M.; Asano, T.; Tanaka, T.; et al. Defucosylated Anti-Epidermal Growth Factor Receptor Monoclonal Antibody 134-mG(2a)-f Exerts Antitumor Activities in Mouse Xenograft Models of Dog Epidermal Growth Factor Receptor-Overexpressed Cells. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 177-183, doi:10.1089/mab.2021.0022.
42. Takei, J.; Kaneko, M.K.; Ohishi, T.; Kawada, M.; Harada, H.; Kato, Y. A novel anti-EGFR monoclonal antibody (EMab-17) exerts antitumor activity against oral squamous cell carcinomas via antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. *Oncol Lett* **2020**, *19*, 2809-2816, doi:10.3892/ol.2020.11384.
43. Tateyama, N.; Asano, T.; Ohishi, T.; Takei, J.; Hosono, H.; Nanamiya, R.; Tanaka, T.; Sano, M.; Saito, M.; Kawada, M.; et al. An Anti-HER2 Monoclonal Antibody H(2)Mab-41 Exerts Antitumor Activities in Mouse Xenograft Model Using Dog HER2-Overexpressed Cells. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 184-190, doi:10.1089/mab.2021.0025.
44. Tanaka, T.; Ohishi, T.; Asano, T.; Takei, J.; Nanamiya, R.; Hosono, H.; Sano, M.; Harada, H.; Kawada, M.; Kaneko, M.K.; et al. An anti-TROP2 monoclonal antibody TrMab-6 exerts antitumor activity in breast cancer mouse xenograft models. *Oncol Rep* **2021**, *46*, 132, doi:10.3892/or.2021.8083.
45. Asano, T.; Ohishi, T.; Takei, J.; Nakamura, T.; Nanamiya, R.; Hosono, H.; Tanaka, T.; Sano, M.; Harada, H.; Kawada, M.; et al. Anti-HER3 monoclonal antibody exerts antitumor activity in a mouse model of colorectal adenocarcinoma. *Oncol Rep* **2021**, *46*, 173, doi:10.3892/or.2021.8124.
46. Kato, Y.; Ohishi, T.; Yamada, S.; Itai, S.; Takei, J.; Sano, M.; Nakamura, T.; Harada, H.; Kawada, M.; Kaneko, M.K. Anti-Human Epidermal Growth Factor Receptor 2 Monoclonal Antibody H(2)Mab-41 Exerts Antitumor Activity in a Mouse Xenograft Model of Colon Cancer. *Monoclon Antib Immunodiagn Immunother* **2019**, *38*, 157-161, doi:10.1089/mab.2019.0017.
47. Gaber, A.; Lenarčič, B.; Pavšič, M. Current View on EpCAM Structural Biology. *Cells* **2020**, *9*, doi:10.3390/cells9061361.
48. Rupp, B.; Ball, H.; Wuchu, F.; Nagrath, D.; Nagrath, S. Circulating tumor cells in precision medicine: challenges and opportunities. *Trends Pharmacol Sci* **2022**, *43*, 378-391, doi:10.1016/j.tips.2022.02.005.
49. Casaletto, J.B.; Geddie, M.L.; Abu-Yousif, A.O.; Masson, K.; Fulgham, A.; Boudot, A.; Maiwald, T.; Kearns, J.D.; Kohli, N.; Su, S.; et al. MM-131, a bispecific anti-Met/EpCAM mAb, inhibits HGF-dependent and HGF-independent Met signaling through concurrent binding to EpCAM. *Proc Natl Acad Sci U S A* **2019**, *116*, 7533-7542, doi:10.1073/pnas.1819085116.

 doi:10.20944/preprints202210.0171.v1

50. Willuda, J.; Honegger, A.; Waibel, R.; Schubiger, P.A.; Stahel, R.; Zangemeister-Wittke, U.; Plückthun, A. High thermal stability is essential for tumor targeting of antibody fragments: engineering of a humanized anti-epithelial glycoprotein-2 (epithelial cell adhesion molecule) single-chain Fv fragment. *Cancer Res* **1999**, *59*, 5758-5767.

51. Geuijen, C.A.W.; De Nardis, C.; Maussang, D.; Rovers, E.; Gallenne, T.; Hendriks, L.J.A.; Visser, T.; Nijhuis, R.; Logtenberg, T.; de Kruif, J.; et al. Unbiased Combinatorial Screening Identifies a Bispecific IgG1 that Potently Inhibits HER3 Signaling via HER2-Guided Ligand Blockade. *Cancer Cell* **2018**, *33*, 922-936.e910, doi:10.1016/j.ccell.2018.04.003.

52. Schram, A.M.; Odintsov, I.; Espinosa-Cotton, M.; Khodos, I.; Sisso, W.J.; Mattar, M.S.; Lui, A.J.W.; Vojnic, M.; Shameem, S.H.; Chauhan, T.; et al. Zenocutuzumab, a HER2xHER3 Bispecific Antibody, Is Effective Therapy for Tumors Driven by NRG1 Gene Rearrangements. *Cancer Discov* **2022**, *12*, 1233-1247, doi:10.1158/2159-8290.Cd-21-1119.