

Article

Development of A Novel Anti-EpCAM Monoclonal Antibody for Various Applications

Guanjie Li¹, Hiroyuki Suzuki^{1,*}, Teizo Asano², Tomohiro Tanaka², Hiroyoshi Suzuki³, Mika K. Kaneko² and Yukinari Kato^{1,2,*}

¹ Department of Molecular Pharmacology, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, Miyagi 980-8575, Japan; kanketsu@med.tohoku.ac.jp (G.L.);

² Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, Miyagi 980-8575, Japan; tomohiro.tanaka.b5@tohoku.ac.jp (T.T.); teizo.asano.a7@tohoku.ac.jp (T.A.); k.mika@med.tohoku.ac.jp (M.K.K.)

³ Department of Pathology and Laboratory Medicine, Sendai Medical Center, 2-11-12, Miyagino, Miyagino-ku, Sendai, Miyagi 983-0045, Japan; hirosuzsnh@gmail.com (Hiroyoshi S.)

* Correspondence: hiroyuki.suzuki.b4@tohoku.ac.jp (Hiroyuki S.); yukinarikato@med.tohoku.ac.jp (Y.K.); Tel.: +81-22-717-8207

Abstract: The epithelial cell adhesion molecule (EpCAM) is a cell surface glycoprotein, which is widely expressed on normal and cancer cells. EpCAM is involved in cell adhesion, proliferation, survival, stemness, and tumorigenesis. Therefore, EpCAM is thought to be a promising target for cancer diagnosis and therapy. In this study, we established anti-EpCAM monoclonal antibodies (mAbs) using the Cell-Based Immunization and Screening (CBIS) method. We characterized them using flow cytometry, western blotting, and immunohistochemistry. One of the established recombinant anti-EpCAM mAbs, recEpMab-37 (mouse IgG₁, kappa), reacted with EpCAM-overexpressed Chinese hamster ovary-K1 cells (CHO/EpCAM) or a colorectal carcinoma cell line (Caco-2). In contrast, recEpMab-37 did not react with EpCAM-knocked out Caco-2 cells. The K_D of recEpMab-37 for CHO/EpCAM and Caco-2 was 2.0×10^{-8} M and 3.2×10^{-8} M, respectively. In western blot analysis, recEpMab-37 detected EpCAM of CHO/EpCAM and Caco-2 cells. Furthermore, recEpMab-37 could stain formalin-fixed paraffin-embedded colorectal carcinoma tissues by immunohistochemistry. Taken together, recEpMab-37, established by CBIS method, is useful for detecting EpCAM in various applications.

Keywords: EpCAM; monoclonal antibody; recombinant antibody; colorectal carcinoma

1. Introduction

The epithelial cell adhesion molecule (EpCAM) is an approximately 40 kilodalton (kDa), type I transmembrane glycoprotein, which is expressed on the basolateral membrane of most epithelial cells [1]. EpCAM is a unique cell adhesion molecule that does not resemble other classical adhesion molecules (e.g., cadherins, selectins, and integrins) in either structure or function. EpCAM forms homophilic and intercellular adhesion, which plays critical roles in the maintenance the epithelial integrity [2]. EpCAM also serves as a signaling molecule. Upon cleavage of EpCAM at the cell membrane, the intracellular domain of EpCAM serves as a transcriptional cofactor with β -catenin and regulates the transcription of target genes involved with cell proliferation, survival, and the maintenance of stem cell property [3].

In addition to the role in normal epithelial tissues, EpCAM plays an important role in tumor development, and was the first identified a human tumor-associated antigen [4]. Studies have shown that EpCAM is overexpressed in the many epithelial cancers including pancreas, colorectal, and prostate cancers [5]. Due to the high expression and antigenicity, EpCAM has been a target of monoclonal antibody (mAb) therapies in colorectal cancer [6,7]. More recently, a bispecific EpCAM/CD3-antibody, Catumaxomab, serve as the

trifunctional antibody. Catumaxomab binds to EpCAM-overexpressed tumor and recruits T cells to tumor cells, which promote cytotoxic synapse formation and tumor cell lysis. Furthermore, through the Fc domain, Catumaxomab also recruits natural killer cells and macrophages and enhances the antibody-dependent cellular cytotoxicity (ADCC) [8,9]. Several clinical trials have demonstrated promising outcomes [10-12], and Catumaxomab has been approved by the European Union for the treatment of patients with malignant ascites in EpCAM-positive carcinomas [13].

Moreover, anti-EpCAM mAbs are most widely used to collect circulating tumor cells (CTCs), which are useful indicators of micro-metastasis and provides important prognostic information to determine the response to therapeutic interventions [14]. The clinical importance of CTCs was confirmed by the U.S. Food and Drug Administration (FDA) approval of CellSearch, a platform that could extract EpCAM-positive, CD45-negative cells from whole blood samples [15]. Therefore, the establishment of anti-EpCAM mAbs is important to improve the efficacy of above applications and to develop novel modalities for tumor therapy. In this study, we developed anti-EpCAM mAbs using Cell-Based Immunization and Screening (CBIS) method [16-31], and evaluated it for various applications, including flow cytometry, western blotting, and immunohistochemical analyses.

2. Materials and Methods

2.1 Cell lines

P3X63Ag8U.1 (P3U1), CHO-K1, and Caco-2 (human colorectal carcinoma) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). CHO/EpCAM was established by transfecting pCAG/EpCAM-PA into CHO-K1 cells using the Neon Transfection System (Thermo Fisher Scientific, Inc., Waltham, MA, USA) as described previously [32]. EpCAM-knocked out Caco-2 (BINDS-16) was established as described previously [33], and available from Tohoku University Cell Bank (Kato's Lab; http://www.med-tohoku-antibody.com/topics/001_paper_cell.htm). P3U1, CHO-K1, and CHO/EpCAM were cultured in RPMI 1640 media (Nacalai Tesque, Inc., Kyoto, Japan). Caco-2 was cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc.). The media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc.), 100 U/ml of penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Nacalai Tesque, Inc.). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

2.2. Animals

A BALB/c mouse was purchased from CLEA Japan (Tokyo, Japan). The animal was housed under specific pathogen-free conditions. All animal experiments were conducted following the relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2019NiA-001). Mice was monitored daily for health during the four full weeks duration of the experiment. A reduction of more than 25% of the total body weight was defined as a humane endpoint. The mouse was euthanized by cervical dislocation, and death was verified by respiratory and cardiac arrest.

2.3. Hybridoma production

We used the CBIS method to develop mAbs against EpCAM. Briefly, one BALB/c mouse was intraperitoneally (i.p.) immunized with CHO/EpCAM cells (1×10^8) together with Imject Alum adjuvant (Thermo Fisher Scientific Inc.). The procedure included three additional immunizations, followed by a final booster injection administered i.p. two days before spleen cell harvesting. Spleen cells were then fused with P3U1 cells using PEG1500

(Roche Diagnostics, Indianapolis, IN, USA). Hybridomas were grown in RPMI 1640, supplemented with 10% FBS, 100 U/ml of penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Hypoxanthine, aminopterin, and thymidine (HAT; Thermo Fisher Scientific Inc.) were also added for hybridoma selection. Culture supernatants were screened using flow cytometry.

2.4. Production of the recombinant antibody

Variable (V_H) and constant (C_H) regions of heavy chain cDNAs of EpMab-37 were subcloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) along with variable (V_L) and constant (C_L) regions of light chain cDNAs of EpMab-37 into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation) to produce recombinant EpMab-37 (recEpMab-37). EpMab-37 vectors were transfected into ExpiCHO-S cells using the ExpiCHO Expression System (Thermo Fisher Scientific Inc.). recEpMab-37 was purified using Ab-Capcher (ProteNova, Kagawa, Japan).

2.5 Flow cytometry

CHO/EpCAM, CHO-K1, Caco-2, and BINDS-16 cells were harvested after brief exposure to 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), cells were treated with recEpMab-37 for 30 min at 4°C, and then with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA). Fluorescence data were collected using SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

2.6 Determination of the binding affinity

CHO/EpCAM and Caco-2 cells were suspended in 100 µl of serially diluted recEpMab-37 (6 ng/ml–100 µg/ml), followed by the addition of Alexa Fluor 488-conjugated anti-mouse IgG (1:200). Fluorescence data were collected using BD FACSLyric (BD Biosciences, Franklin Lakes, NJ). 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, USA).

2.7 Western blot analysis

Cell lysates of CHO/EpCAM, CHO-K1, Caco-2, and BINDS-16 cells were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.). The samples were then electrophoresed on 5–20% polyacrylamide gels (Nacalai Tesque, Inc.) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.) for 1 h, the membrane was incubated with recEpMab-37 (1 µg/ml) or anti-β-actin (1 µg/ml) for 1 h, followed by incubation with HRP-conjugated anti-mouse immunoglobulins (Agilent Technologies, Inc., Santa Clara, CA, USA) at a 1:2,000 dilution for 1 h. The membrane was developed using the ImmunoStar LD Chemiluminescence Reagent (FUJIFILM Wako Pure Chemical Corporation) and a Sayaca-Imager (DRC Co., Ltd., Tokyo, Japan). All Western blot procedures were performed at room temperature.

2.8 Immunohistochemical analysis

Histologic sections 4 µm thick of colorectal carcinoma tissue array (Catalog number: CO243b; US Biomax Inc., Rockville, MD, USA) were autoclaved in citrate buffer (pH 6.0; Agilent Technologies Inc.) for 20 min. Sections were then incubated with 1 µg/ml of re-

cEpMab-37 for 1 h at room temperature and treated using an Envision+ kit (Agilent Technologies, Inc.) for 30 min. The color was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 2 min, and sections were then counterstained with hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

3. Results

3.1 Flow cytometric analysis of recEpMab-37 to EpCAM-expressing cells

In this study, EpMab-37 was established by immunizing one mouse with CHO/EpCAM cells (Fig. 1). We have confirmed the reactivity of EpMab-37 to CHO/EpCAM cells, cloned the cDNAs of EpMab-37, and produced the recombinant mAb, recEpMab-37.

We first confirmed the reactivity of recEpMab-37 against CHO/EpCAM by flow cytometry. As shown in Fig. 2A, recEpMab-37 recognized CHO/EpCAM cells in a dose-dependent manner, but not CHO-K1 cells (Fig. 2B). We next examined whether recEpMab-37 could recognize endogenous EpCAM in colorectal carcinoma Caco-2 cells. recEpMab-37 reacted with Caco-2 cells in a dose-dependent manner (Fig. 2C). However, recEpMab-37 never reacted with EpCAM-knocked out Caco-2 (BINDS-16) cells (Fig. 2D).

Then, we determined the apparent binding affinity of recEpMab-37 with CHO/EpCAM and Caco-2 using flow cytometry. The K_D of recEpMab-37 for CHO/EpCAM and Caco-2 was 2.0×10^{-8} M and 3.2×10^{-8} M, respectively, indicating that recEpMab-37 possesses the moderate affinity for EpCAM-expressing cells (Fig. 3).

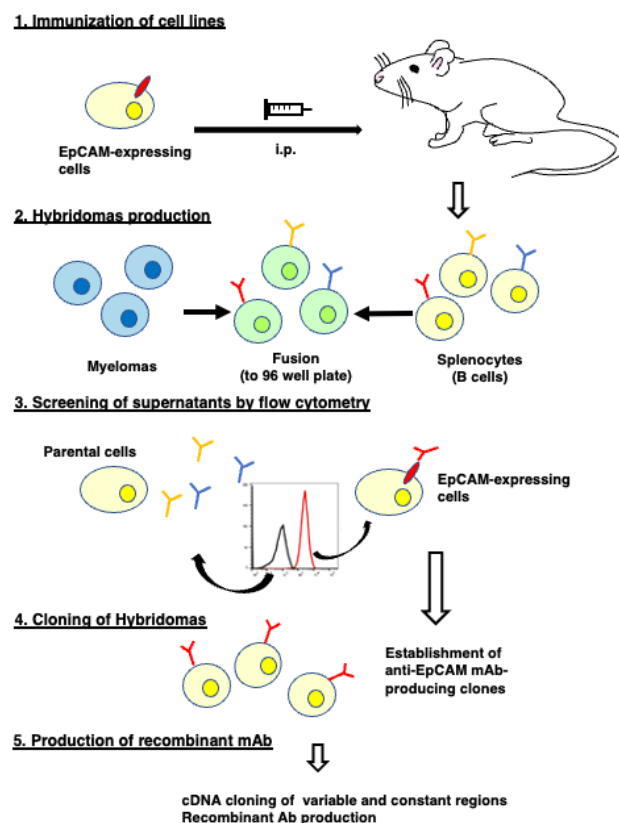


Figure 1. A schematic procedure of anti-EpCAM mAbs production. A mouse was intraperitoneally immunized with the CHO/EpCAM cells. The screening was then conducted by flow cytometry using parental cells and EpCAM-overexpressed CHO-K1 cells. Variable and constant regions cDNAs were cloned from an established clone, and the recombinant Ab was produced.

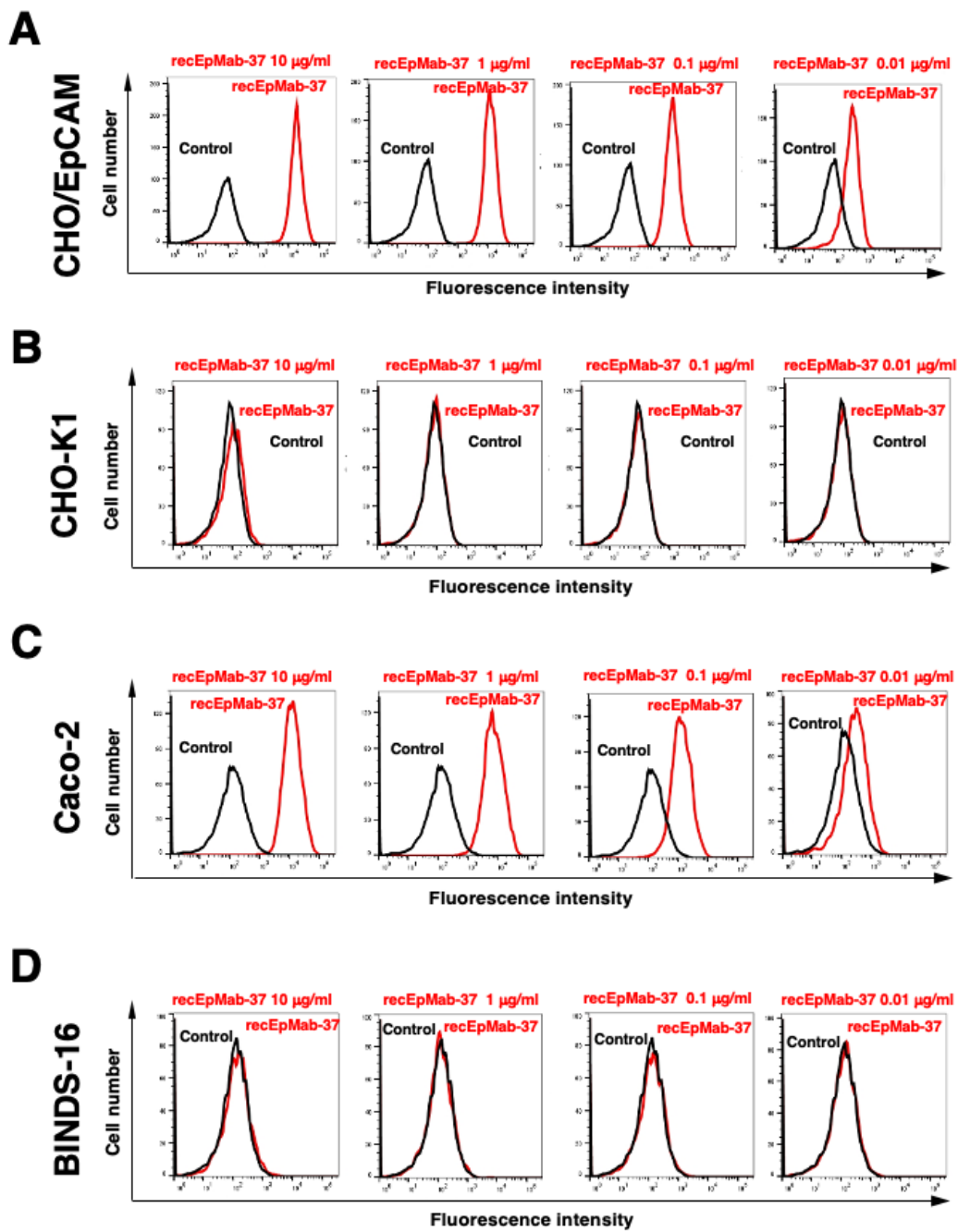


Figure 2. Flow cytometry to EpCAM expressing cells using recEpMab-37. CHO/EpCAM (A), CHO-K1 (B), Caco-2 (C), and BINDS-16 (EpCAM-knocked out Caco-2) (D) cells were treated with 0.01–10 µg/mL of recEpMab-37, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Black line represents the negative control.

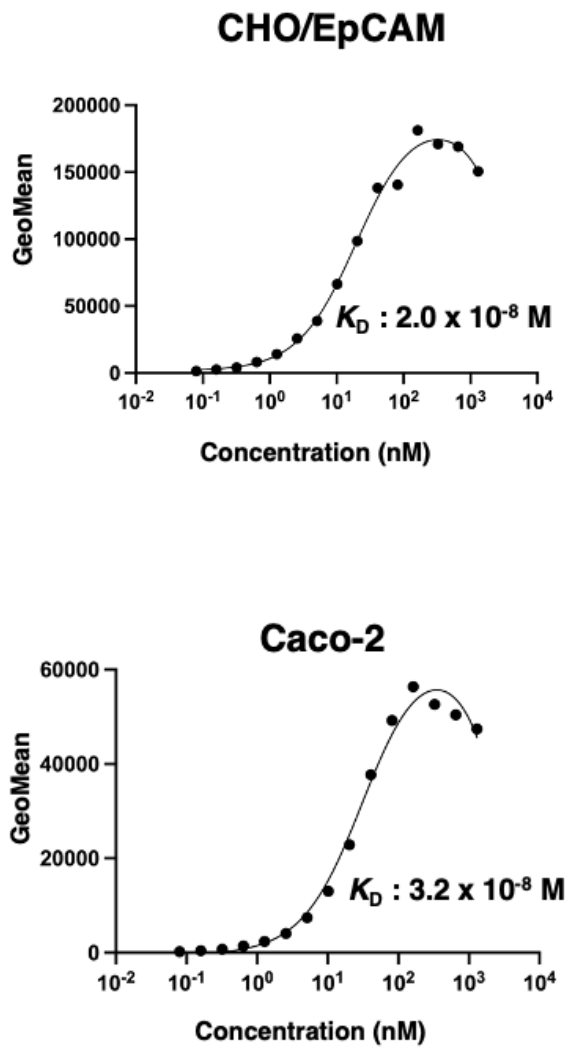


Figure 3. The determination of the binding affinity of recEpMab-37. CHO/EpCAM and Caco-2 cells were suspended in 100 μ L serially diluted recEpMab-37 (6 ng/mL to 100 μ g/mL). Then, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were subsequently collected using a BD FACSLytic, following the calculation of the dissociation constant (K_D) by GraphPad PRISM 8.

3.2 Western blot analysis

Western blotting was performed to further assess the sensitivity of recEpMab-37. Lysates of CHO-K1 and CHO/EpCAM cells were probed. As shown in Fig. 4, recEpMab-37 detected EpCAM as 35 ~ 40 kDa double bands. However, recEpMab-37 did not detect any bands from lysates of CHO-K1 cells. These results indicate that recEpMab-37 specifically detects exogenous EpCAM. Furthermore, recEpMab-37 could detect endogenous EpCAM as a 35-kDa band from lysates of Caco-2 cells. In contrast, recEpMab-37 could not detect any bands from lysates of EpCAM-knocked out Caco-2 (BINDS-16) cells. These results suggest that recEpMab-37 specifically recognizes endogenous EpCAM in the colorectal carcinoma cell line.

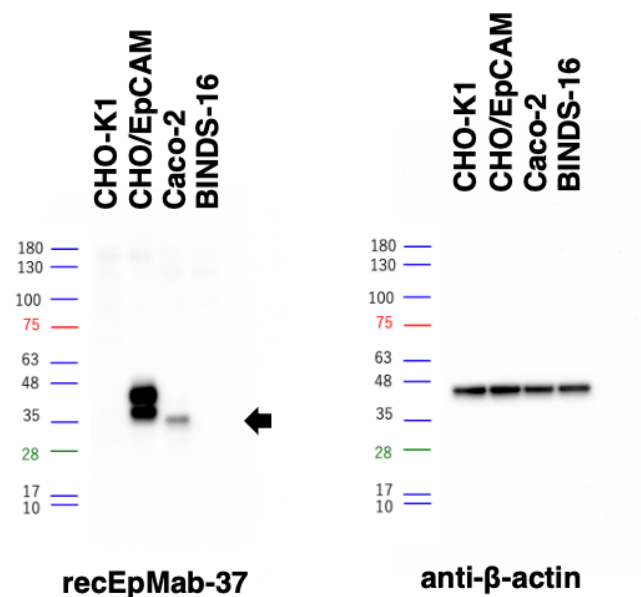


Figure 4. Western blotting by recEpMab-37. Cell lysates of CHO-K1, CHO/EpCAM, Caco-2, and BINDS-16 (10 µg) were electrophoresed and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with 1 µg/mL of recEpMab-37 and 1 µg/mL of anti-β-actin and subsequently with peroxidase-conjugated anti-mouse immunoglobulins. Black arrows indicate the predicted size of EpCAM (~35 kDa).

3.3 Immunohistochemical analysis using recEpMab-37 against colorectal carcinoma tissues.

We investigated whether recEpMab-37 can be applicable for immunohistochemical analyses using formalin-fixed paraffin-embedded (FFPE) colorectal carcinoma sections. recEpMab-37 strongly stained colorectal carcinoma cells in a colorectal carcinoma section (Fig. 5A and 5B), but not stromal tissues (Fig. 5A). A cytoplasmic staining pattern in carcinoma cells was observed (Fig. 5B). Hematoxylin and eosin (HE) staining was performed using the serial sections (Fig. 5C and 5D). In contrast, recEpMab-37 did not stain normal colorectal epithelium (Fig. 5E and 5F). HE staining was also performed using the serial sections (Fig. 5G and 5H). These results indicate that recEpMab-37 is useful for immunohistochemical analysis of FFPE tumor sections.

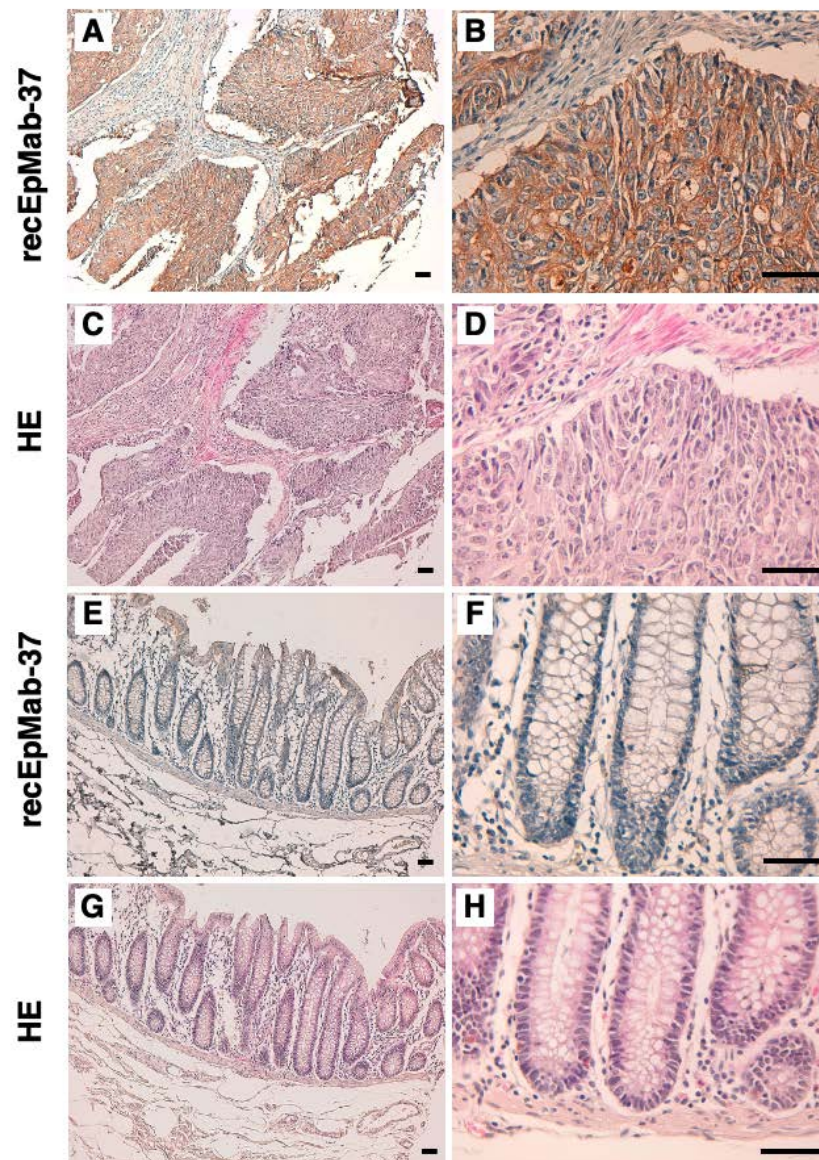


Figure 5. Immunohistochemical analysis using recEpMab-37 against colorectal carcinoma tissues. (A, B, E, F) After antigen retrieval, sections were incubated with 1 $\mu\text{g/mL}$ of recEpMab-37 followed by treatment with the Envision+ kit. Color was developed using DAB for 2 min, and sections were counterstained with hematoxylin. (C, D, G, H) Hematoxylin and eosin (HE) staining. Scale bar = 100 μm .

4. Discussion

In this study, we first developed EpMab-37 using the CBIS method and next produced its recombinant mAb (recEpMab-37) (Fig. 1). Then, we investigated the usefulness of recEpMab-37 for flow cytometry (Fig. 2), western blotting (Fig. 4), and immunohistochemistry (Fig. 5). Since recEpMab-37 does not detect any bands in EpCAM-knocked out Caco-2 cells, recEpMab-37 exhibited the high specificity to EpCAM. Further studies are warranted to determine the binding epitope of EpMab-37. We could not determine the epitope of EpMab-37 using conventional peptides scanning of EpCAM extracellular domain (data not shown). Because EpMab-37 was established by immunization of EpCAM-overexpressed CHO-K1 cells, EpMab-37 could recognize the native and conformational epitope. We have developed the Arg, Ile, Glu, Asp, and Leu (RIEDL)-insertion for epitope mapping (REMAP) and His-tag insertion for epitope mapping (HisMAP) method to determine the conformational epitopes. We have successfully determined the epitopes of anti-EGFR mAbs (EMab-51 and EMab-134) [34,35] and anti-CD44 mAbs (C₄₄Mab-5 and C₄₄Mab-46) [36,37] by the REMAP method, and anti-CD20 mAbs (C₂₀Mab-11 and C₂₀Mab-60) by HisMAP method [38,39]. The determination of EpMab-37 epitope may provide useful information for future applications.

EpCAM plays critical roles in tumorigenesis to promote tumor cell proliferation, survival, and tumor-initiating potential [2]. EpCAM has been reported to be overexpressed in several tumors [40-44], which correlates with poor prognosis, therapeutic failure, and early tumor recurrence [43]. Therefore, EpCAM has been considered as a promising therapeutic target. A humanized scFv against EpCAM, fused to Pseudomonas exotoxin A, Oportuzumab monatox (Vicinium, VB4-845), has been developed [3]. After Vicinium targets EpCAM on tumor cells, it is internalized, receives the cleavage of the linker, releases the payload exotoxin, and exhibits the cytotoxicity. Vicinium was granted the FDA fast track designation for the treatment of bacillus Calmette-Guérin (BCG)-unresponsive non-muscle invasive bladder cancer (NMIBC) [45]. Vicinium was evaluated in the single-arm Phase 3 VISTA study (NCT02449239) for the treatment of patients with high-grade NMIBC *in situ* and high-grade papillary disease previously treated with BCG. However, FDA did not approve Vicinium for BCG-unresponsive NMIBC in August 2021 [46].

As mentioned above, clinical applications of anti-EpCAM mAbs are still limited since anti-EpCAM mAbs might generate a degree of toxicity against normal tissues. We previously established cancer-specific mAbs (CasMabs) targeting to podoplanin [47-50] and podocalyxin [51], which are expressed on many tumors [52-55]. It is worthwhile to develop cancer-specific anti-EpCAM mAbs using CasMab method. We previously converted a mouse IgG₁ subclass into a mouse IgG_{2a}, and produced a defucosylated version which exhibited high ADCC activities *in vitro*, and significantly reduced tumor growth [56]. Therefore, the production of a class-switched and defucosylated version of EpMab-37 is warranted to evaluate the *in vivo* antitumor activity.

Author Contributions: G.L., T.T., and T.A. performed the experiments. Hiroyoshi S., M.K.K., and Y.K. designed the experiments. G.L. analyzed the data. G.L., Hiroyuki S. and Y.K. wrote the manuscript.

Funding: This research was supported in part by Japan Agency for Medical Research and Development (AMED) under Grant Numbers: JP22ama121008 (to Y.K.), JP21am0401013 (to Y.K.), and JP21am0101078 (to Y.K.).

Institutional Review Board Statement: The animal study protocol was approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2019NiA-001) for studies involving animals.

Conflicts of Interest: The authors declare no conflicts 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*, doi:10.3390/biom11070956.
3. 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.
4. Herlyn, M.; Stepilewski, 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.
5. Went, P.T.; Lugli, A.; Meier, S.; Bundi, M.; Mirlacher, M.; Sauter, G.; Dirnhofer, S. Frequent EpCam protein expression in human carcinomas. *Hum Pathol* **2004**, *35*, 122-128, doi:10.1016/j.humpath.2003.08.026.
6. Riethmüller, G.; Holz, E.; Schlimok, G.; Schmiegel, W.; Raab, R.; Höffken, K.; Gruber, R.; Funke, I.; Pichlmaier, H.; Hirche, H.; et al. Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. *J Clin Oncol* **1998**, *16*, 1788-1794, doi:10.1200/jco.1998.16.5.1788.
7. Sears, H.F.; Atkinson, B.; Mattis, J.; Ernst, C.; Herlyn, D.; Stepilewski, 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.
8. 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*, doi:10.3390/cancers12051279.
9. Zhang, X.; Yang, Y.; Fan, D.; Xiong, D. The development of bispecific antibodies and their applications in tumor immune escape. *Exp Hematol Oncol* **2017**, *6*, 12, doi:10.1186/s40164-017-0072-7.
10. 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.
11. 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.
12. 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.
13. Linke, R.; Klein, A.; Seimetz, D. Catumaxomab: clinical development and future directions. *MAbs* **2010**, *2*, 129-136, doi:10.4161/mabs.2.2.11221.
14. 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.
15. 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.

16. Tanaka, T.; Nanamiya, R.; Takei, J.; Nakamura, T.; Yanaka, M.; Hosono, H.; Sano, M.; Asano, T.; Kaneko, M.K.; Kato, Y. Development of Anti-Mouse CC Chemokine Receptor 8 Monoclonal Antibodies for Flow Cytometry. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 65-70, doi:10.1089/mab.2021.0005.
17. Tanaka, T.; Asano, T.; Sano, M.; Takei, J.; Hosono, H.; Nanamiya, R.; Nakamura, T.; Yanaka, M.; Harada, H.; Fukui, M.; et al. Development of Monoclonal Antibody PMab-269 Against California Sea Lion Podoplanin. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 124-133, doi:10.1089/mab.2021.0011.
18. Takei, J.; Asano, T.; Nanamiya, R.; Nakamura, T.; Yanaka, M.; Hosono, H.; Tanaka, T.; Sano, M.; Kaneko, M.K.; Harada, H.; et al. Development of Anti-human T Cell Immunoreceptor with Ig and ITIM Domains (TIGIT) Monoclonal Antibodies for Flow Cytometry. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 71-75, doi:10.1089/mab.2021.0006.
19. Sayama, Y.; Kaneko, M.K.; Takei, J.; Hosono, H.; Sano, M.; Asano, T.; Kato, Y. Establishment of a novel anti-TROP2 monoclonal antibody TrMab-29 for immunohistochemical analysis. *Biochem Biophys Rep* **2021**, *25*, 100902, doi:10.1016/j.bbrep.2020.100902.
20. Sayama, Y.; Kaneko, M.K.; Kato, Y. Development and characterization of TrMab-6, a novel anti-TROP2 monoclonal antibody for antigen detection in breast cancer. *Mol Med Rep* **2021**, *23*, doi:10.3892/mmr.2020.11731.
21. Nanamiya, R.; Takei, J.; Asano, T.; Tanaka, T.; Sano, M.; Nakamura, T.; Yanaka, M.; Hosono, H.; Kaneko, M.K.; Kato, Y. Development of Anti-Human CC Chemokine Receptor 9 Monoclonal Antibodies for Flow Cytometry. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 101-106, doi:10.1089/mab.2021.0007.
22. Hosono, H.; Asano, T.; Takei, J.; Sano, M.; Tanaka, T.; Kaneko, M.K.; Kato, Y. Development of an Anti-Elephant Podoplanin Monoclonal Antibody PMab-265 for Flow Cytometry. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 141-145, doi:10.1089/mab.2021.0015.
23. Asano, T.; Nanamiya, R.; Takei, J.; Nakamura, T.; Yanaka, M.; Hosono, H.; Tanaka, T.; Sano, M.; Kaneko, M.K.; Kato, Y. Development of Anti-Mouse CC Chemokine Receptor 3 Monoclonal Antibodies for Flow Cytometry. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 107-112, doi:10.1089/mab.2021.0009.
24. Kaneko, M.K.; Sano, M.; Takei, J.; Asano, T.; Sayama, Y.; Hosono, H.; Kobayashi, A.; Konnai, S.; Kato, Y. Development and Characterization of Anti-Sheep Podoplanin Monoclonal Antibodies PMab-253 and PMab-260. *Monoclon Antib Immunodiagn Immunother* **2020**, *39*, 144-155, doi:10.1089/mab.2020.0018.
25. Furusawa, Y.; Kaneko, M.K.; Kato, Y. Establishment of C(20)Mab-11, a novel anti-CD20 monoclonal antibody, for the detection of B cells. *Oncol Lett* **2020**, *20*, 1961-1967, doi:10.3892/ol.2020.11753.
26. Furusawa, Y.; Kaneko, M.K.; Kato, Y. Establishment of an Anti-CD20 Monoclonal Antibody (C(20)Mab-60) for Immunohistochemical Analyses. *Monoclon Antib Immunodiagn Immunother* **2020**, *39*, 112-116, doi:10.1089/mab.2020.0015.
27. Kato, Y.; Furusawa, Y.; Yamada, S.; Itai, S.; Takei, J.; Sano, M.; Kaneko, M.K. Establishment of a monoclonal antibody PMab-225 against alpaca podoplanin for immunohistochemical analyses. *Biochem Biophys Rep* **2019**, *18*, 100633, doi:10.1016/j.bbrep.2019.100633.
28. Furusawa, Y.; Yamada, S.; Itai, S.; Nakamura, T.; Yanaka, M.; Sano, M.; Harada, H.; Fukui, M.; Kaneko, M.K.; Kato, Y. PMab-219: a monoclonal antibody for the immunohistochemical analysis of horse podoplanin. *Biochem. Biophys. Rep.* **2019**, *18*, 100616, doi:10.1016/j.bbrep.2019.01.009.
29. Yamada, S.; Itai, S.; Nakamura, T.; Yanaka, M.; Kaneko, M.K.; Kato, Y. Detection of high CD44 expression in oral cancers using the novel monoclonal antibody, C(44)Mab-5. *Biochem Biophys Rep* **2018**, *14*, 64-68, doi:10.1016/j.bbrep.2018.03.007.

30. Yamada, S.; Itai, S.; Nakamura, T.; Yanaka, M.; Chang, Y.W.; Suzuki, H.; Kaneko, M.K.; Kato, Y. Monoclonal Antibody L(1)Mab-13 Detected Human PD-L1 in Lung Cancers. *Monoclon Antib Immunodiagn Immunother* **2018**, *37*, 110-115, doi:10.1089/mab.2018.0004.
31. Itai, S.; Yamada, S.; Kaneko, M.K.; Harada, H.; Kagawa, Y.; Konnai, S.; Kato, Y. Expression of Cat Podoplanin in Feline Squamous Cell Carcinomas. *Monoclon Antib Immunodiagn Immunother* **2017**, *36*, 243-250, doi:10.1089/mab.2017.0046.
32. Kaneko, M.K.; Ohishi, T.; Takei, J.; Sano, M.; Nakamura, T.; Hosono, H.; Yanaka, M.; Asano, T.; Sayama, Y.; Harada, H.; et al. Anti-EpCAM monoclonal antibody exerts antitumor activity against oral squamous cell carcinomas. *Oncol Rep* **2020**, *44*, 2517-2526, doi:10.3892/or.2020.7808.
33. Hosono, H.; Ohishi, T.; Takei, J.; Asano, T.; Sayama, Y.; Kawada, M.; Kaneko, M.K.; Kato, Y. The anti-epithelial cell adhesion molecule (EpCAM) monoclonal antibody EpMab-16 exerts antitumor activity in a mouse model of colorectal adenocarcinoma. *Oncol Lett* **2020**, *20*, 383, doi:10.3892/ol.2020.12246.
34. Sano, M.; Kaneko, M.K.; Asano, T.; Kato, Y. Epitope Mapping of an Antihuman EGFR Monoclonal Antibody (EMab-134) Using the REMAP Method. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 191-195, doi:10.1089/mab.2021.0014.
35. Nanamiya, R.; Sano, M.; Asano, T.; Yanaka, M.; Nakamura, T.; Saito, M.; Tanaka, T.; Hosono, H.; Tateyama, N.; Kaneko, M.K.; et al. Epitope Mapping of an Anti-Human Epidermal Growth Factor Receptor Monoclonal Antibody (EMab-51) Using the RIEDL Insertion for Epitope Mapping Method. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 149-155, doi:10.1089/mab.2021.0010.
36. Asano, T.; Kaneko, M.K.; Kato, Y. Development of a Novel Epitope Mapping System: RIEDL Insertion for Epitope Mapping Method. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 162-167, doi:10.1089/mab.2021.0023.
37. Asano, T.; Kaneko, M.K.; Takei, J.; Tateyama, N.; Kato, Y. Epitope Mapping of the Anti-CD44 Monoclonal Antibody (C44Mab-46) Using the REMAP Method. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 156-161, doi:10.1089/mab.2021.0012.
38. Suzuki, H.; Asano, T.; Tanaka, T.; Kaneko, M.K.; Kato, Y. Epitope Mapping of the Anti-CD20 Monoclonal Antibodies (C20Mab-11 and 2H7) Using HisMAP Method. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 20-26, doi:10.1089/mab.2021.0051.
39. Asano, T.; Suzuki, H.; Kaneko, M.K.; Kato, Y. Epitope Mapping of Rituximab Using HisMAP Method. *Monoclon Antib Immunodiagn Immunother* **2022**, *41*, 8-14, doi:10.1089/mab.2021.0044.
40. Jing, Y.; Zhou, L.; Chen, J.; Xu, H.; Sun, J.; Cai, M.; Jiang, J.; Gao, J.; Wang, H. Quantitatively Mapping the Assembly Pattern of EpCAM on Cell Membranes with Peptide Probes. *Anal Chem* **2020**, *92*, 1865-1873, doi:10.1021/acs.analchem.9b03901.
41. Vorobyeva, A.; Konovalova, E.; Xu, T.; Schulga, A.; Altai, M.; Garousi, J.; Rinne, S.S.; Orlova, A.; Tolmachev, V.; Deyev, S. Feasibility of Imaging EpCAM Expression in Ovarian Cancer Using Radiolabeled DARPIn Ec1. *Int J Mol Sci* **2020**, *21*, doi:10.3390/ijms21093310.
42. van den Brand, D.; van Lith, S.A.M.; de Jong, J.M.; Gorris, M.A.J.; Palacio-Castaneda, V.; Couwenbergh, S.T.; Goldman, M.R.G.; Ebisch, I.; Massuger, L.F.; Leenders, W.P.J.; et al. EpCAM-Binding DARPins for Targeted Photodynamic Therapy of Ovarian Cancer. *Cancers (Basel)* **2020**, *12*, doi:10.3390/cancers12071762.
43. Pandit, H.; Li, Y.; Zheng, Q.; Guo, W.; Yu, Y.; Li, S.; Martin, R.C.G. Carcinogenetic initiation contributed by EpCAM+ cancer cells in orthotopic HCC models of immunocompetent and athymic mice. *Oncotarget* **2020**, *11*, 2047-2060, doi:10.18632/oncotarget.27454.

44. Zhang, D.; Yang, L.; Liu, X.; Gao, J.; Liu, T.; Yan, Q.; Yang, X. Hypoxia modulates stem cell properties and induces EMT through N-glycosylation of EpCAM in breast cancer cells. *J Cell Physiol* **2020**, *235*, 3626-3633, doi:10.1002/jcp.29252.
45. Kaplon, H.; Muralidharan, M.; Schneider, Z.; Reichert, J.M. Antibodies to watch in 2020. *MAbs* **2020**, *12*, 1703531, doi:10.1080/19420862.2019.1703531.
46. Karlovitch, S. FDA Does Not Approve Vicinium for BCG-unresponsive NMIBC. Available from: <https://www.targetedonc.com/view/fda-does-not-approve-vicinium-for-bcg-unresponsive-nmibc> [Last accessed: March/22/2022]. **2021**.
47. Kaneko, M.K.; Nakamura, T.; Kunita, A.; Fukayama, M.; Abe, S.; Nishioka, Y.; Yamada, S.; Yanaka, M.; Saidoh, N.; Yoshida, K.; et al. ChLpMab-23: Cancer-Specific Human-Mouse Chimeric Anti-Podoplanin Antibody Exhibits Antitumor Activity via Antibody-Dependent Cellular Cytotoxicity. *Monoclon Antib Immunodiagn Immunother* **2017**, *36*, 104-112, doi:10.1089/mab.2017.0014.
48. Kaneko, M.K.; Yamada, S.; Nakamura, T.; Abe, S.; Nishioka, Y.; Kunita, A.; Fukayama, M.; Fujii, Y.; Ogasawara, S.; Kato, Y. Antitumor activity of chLpMab-2, a human-mouse chimeric cancer-specific antihuman podoplanin antibody, via antibody-dependent cellular cytotoxicity. *Cancer Med* **2017**, *6*, 768-777, doi:10.1002/cam4.1049.
49. Kato, Y.; Kaneko, M.K. A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. *Sci Rep* **2014**, *4*, 5924, doi:10.1038/srep05924.
50. Miyazaki, A.; Nakai, H.; Sonoda, T.; Hirohashi, Y.; Kaneko, M.K.; Kato, Y.; Sawa, Y.; Hiratsuka, H. LpMab-23-recognizing cancer-type podoplanin is a novel predictor for a poor prognosis of early stage tongue cancer. *Oncotarget* **2018**, *9*, 21156-21165, doi:10.18632/oncotarget.24986.
51. Kaneko, M.K.; Ohishi, T.; Kawada, M.; Kato, Y. A cancer-specific anti-podocalyxin monoclonal antibody (60-mG(2a)-f) exerts antitumor effects in mouse xenograft models of pancreatic carcinoma. *Biochem Biophys Rep* **2020**, *24*, 100826, doi:10.1016/j.bbrep.2020.100826.
52. Kaneko, M.K.; Itai, S.; Yamada, S.; Kato, Y. 47-mG(2a): A Mouse IgG(2a)-Type of PcMab-47 Useful for Detecting Podocalyxin in Esophageal Cancers by Immunohistochemistry. *Monoclon Antib Immunodiagn Immunother* **2018**, *37*, 158-161, doi:10.1089/mab.2018.0003.
53. Tanaka, M.; Kijima, H.; Shimada, H.; Makuuchi, H.; Ozawa, S.; Inokuchi, S. Expression of podoplanin and vimentin is correlated with prognosis in esophageal squamous cell carcinoma. *Mol Med Rep* **2015**, *12*, 4029-4036, doi:10.3892/mmr.2015.3966.
54. Nakashima, Y.; Yoshinaga, K.; Kitao, H.; Ando, K.; Kimura, Y.; Saeki, H.; Oki, E.; Morita, M.; Kakeji, Y.; Hirahashi, M.; et al. Podoplanin is expressed at the invasive front of esophageal squamous cell carcinomas and is involved in collective cell invasion. *Cancer Sci* **2013**, *104*, 1718-1725, doi:10.1111/cas.12286.
55. Rahadiani, N.; Ikeda, J.; Makino, T.; Tian, T.; Qiu, Y.; Mamat, S.; Wang, Y.; Doki, Y.; Aozasa, K.; Morii, E. Tumorigenic role of podoplanin in esophageal squamous-cell carcinoma. *Ann Surg Oncol* **2010**, *17*, 1311-1323, doi:10.1245/s10434-009-0895-5.
56. 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 antiCD44 monoclonal antibody 5mG2af exerts antitumor effects in mouse xenograft models of oral squamous cell carcinoma. *Oncol Rep* **2020**, *44*, 1949-1960, doi:10.3892/or.2020.7735.