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Article

# A Novel Anti-Cadherin-17 Monoclonal Antibody, Ca<sub>17</sub>Mab-5, for Multiple Applications

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## Abstract

**Background/Objectives:** Cadherin-17 (CDH17, LI-cadherin) is a non-classical cadherin with an atypical structure and unique functions. CDH17 expression is restricted to normal intestinal epithelium. Furthermore, CDH17 functions as an oncoprotein that promotes tumor migration and invasion in colorectal, gastric, and pancreatic cancers. Therefore, CDH17 is an important diagnostic marker and therapeutic target. The CDH17-directed strategies, including monoclonal antibodies (mAbs), bispecific Abs, antibody–drug conjugates (ADCs), and chimeric antigen receptor (CAR) T cells, have been evaluated in preclinical and clinical studies. Therefore, developing mAbs that specifically recognize cell surface-expressing CDH17 is essential for advancing both tumor diagnosis and therapy. **Methods:** Anti-human CDH17 mAbs (named Ca<sub>17</sub>Mabs) were developed by immunizing a mouse with CDH17-overexpressed cells and a high-throughput screening using flow cytometry. **Results:** Among Ca<sub>17</sub>Mabs, a clone, Ca<sub>17</sub>Mab-5 (IgG<sub>1</sub>, κ) specifically recognized CDH17-overexpressed Chinese hamster ovary-K1 (CHO/CDH17) cells with no detectable cross-reactivity to 21 other CDHs by flow cytometry. Ca<sub>17</sub>Mab-5 also detected endogenous CDH17 in human colorectal cancer cell lines, COLO201 and COLO205. The dissociation constant ( $K_D$ ) values of Ca<sub>17</sub>Mab-5 for CHO/CDH17 and COLO205 were estimated as  $1.4 \times 10^{-8}$  M and  $1.3 \times 10^{-8}$  M, respectively. Furthermore, Ca<sub>17</sub>Mab-5 detected endogenous CDH17 by Western blotting. In immunohistochemistry, Ca<sub>17</sub>Mab-5 exhibited clear membranous staining in normal colon epithelium and colorectal cancers. **Conclusion:** Ca<sub>17</sub>Mab-5 is a versatile tool for detecting CDH17 and has potential for tumor diagnosis and therapy.

**Keywords:** cadherin 17; CDH17; monoclonal antibody; cell-based immunization and screening; flow cytometry; immunohistochemistry

## 1. Introduction

Cadherins (CDHs) play crucial roles in cell–cell adhesion and the maintenance of tissue architecture [1]. Classical cadherins have five extracellular cadherin (EC) repeats, a single transmembrane domain, and a cytoplasmic domain with highly conserved binding regions for the armadillo proteins including β-catenin and p120-catenin [1]. The EC repeats, originally identified in the extracellular region of the classical type I cadherin CDH1/E-cadherin [2], bind to Ca<sup>2+</sup> and mediate homophilic interactions that are important for cell sorting into different compartments during organogenesis [3]. In epithelia, CDH1 forms adherens junctions via homophilic interactions, which define the features of epithelial sheets and apical adhesive structures [4]. The ability to interact with cytoplasmic armadillo proteins is a key function of classical CDHs. p120 catenin directly associates

with the membrane-proximal region of the CDH cytoplasmic tail, and  $\beta$ -catenin serves as a scaffold to anchor  $\alpha$ -catenin [5]. The cadherin–catenin complex associates directly or indirectly with signaling molecules, scaffolding proteins and cytoskeletal regulators such as filamentous actin [5].

Cadherin-17 (CDH17), also known as liver-intestine CDH (LI-CDH) or seven-domain (7D) CDH, is a non-classical CDH with seven EC repeats, a single transmembrane domain, and a short cytoplasmic domain that lacks armadillo protein-binding motifs [6–8]. The tissue distribution of CDH17 varies across species. CDH17 is expressed in the liver and intestine in rats. In mice and humans, expression is restricted to small-intestinal and colonic epithelial cells, with no detectable expression in the liver or stomach [9].

CDH17 plays a crucial role in intestinal barrier function [6]. CDH17 KO mice displayed enhanced permeability in the small intestine and colon *in vitro* and *in vivo* models. Furthermore, loss of CDH17 resulted in increased susceptibility to dextran sulfate sodium (DSS)-induced colitis [10]. In azoxymethane and DSS-induced colorectal cancer model, CDH17 KO enhanced tumor formation and progression in the intestine [10]. Therefore, CDH17 is essential for intestinal homeostasis by limiting intestinal epithelial permeability and serves as a tumor suppressor in colitis-associated tumor development.

Meanwhile, CDH17 is upregulated in colorectal cancer [11] and has been identified as a key mediator of metastatic progression and poor patient survival [12] through interactions with unique binding partners [13,14]. CDH17 binds to the desmosomal cadherin desmocollin-1 (DSC1) and, via the DSC1/p120-catenin complex, indirectly interacts with actin filaments to promote migration and invasiveness in colorectal cancer [15]. CDH17 also interacts with  $\alpha 2\beta 1$  integrin via the RGD motif in the ectodomain, promoting colorectal cancer liver metastasis [12,16]. Furthermore, a global transcriptome analysis revealed that CDH17 silencing in metastatic colorectal cancer cell lines resulted in a marked reduction of the intestinal cancer stem cell marker LGR5, which led to attenuation of Wnt/ $\beta$ -catenin signaling, suppression of stemness-related genes, and consequent impairment of stem cell phenotypes [17].

CDH17 has been considered a promising therapeutic target for preventing metastatic progression in colorectal cancer, with approaches, including monoclonal antibodies (mAbs), bispecific Abs, antibody–drug conjugates (ADCs), and chimeric antigen receptor (CAR)-T [18,19]. CDH17-targeting ADCs have been developed and evaluated in clinical studies. TORL-3-600 is a humanized ADC with an MMAE payload that entered a phase I clinical trial for the treatment of advanced colorectal cancer (NCT05948826) [20]. AMT-676 [21] and YL217 [22] are humanized ADCs with topoisomerase I inhibitor payloads that have entered phase I clinical trials for the treatment of advanced solid tumors (NCT06400485 and NCT06859762, respectively). Furthermore, a T-cell engager cabotamig (CD3  $\times$  CDH17 bispecific Ab, NCT05411133) and anti-CDH17 CAR-T therapies (NCT06055439, NCT06501183, and NCT07216560) have been evaluated in clinical studies.

MABs that detect CDH17 have been developed for Western blotting or immunohistochemistry (IHC). However, suitable anti-CDH17 mAbs for flow cytometry and IHC have been limited. We have developed the Cell-Based Immunization and Screening (CBIS), which includes immunizing antigen-overexpressed cells and high-throughput flow cytometry–based screening to generate highly versatile and specific mAbs. Using the CBIS method, we have developed anti-CDH1 [23], anti-CDH5/VE-cadherin [24], anti-CDH13 [25], and anti-CDH15/M-cadherin [26] mAbs for flow cytometry, Western blotting, and IHC. MABs obtained from the CBIS method typically recognize conformational epitopes, enabling their use in flow cytometry. Notably, some of these mAbs are also used in Western blotting and IHC. In this study, we employed the CBIS method to develop highly versatile anti-CDH17 mAbs.

## 2. Materials and Methods

### 2.1. Cell Lines

Human glioblastoma (GBM) LN229, human colorectal cancer COLO205 and HCT116, Chinese hamster ovary (CHO)-K1, and mouse myeloma P3X63Ag8U.1 (P3U1) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Another human colorectal cancer cell line, COLO201, was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Miyagi, Japan).

CHO-K1, P3U1, and CDH-overexpressed CHO-K1 (e.g., CHO/CDH1), COLO201, and COLO205 were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Nacalai Tesque, Inc.). LN229 and CDH17-overexpressed LN229 (LN229/CDH17) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Nacalai Tesque, Inc.) supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific, Inc.), 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Nacalai Tesque, Inc.). Cells were then maintained in a humidified CO<sub>2</sub> incubator at 37 °C with 5% CO<sub>2</sub> and 95% air.

### 2.2. Establishment of Cadherin-Overexpressed Stable Transfectants

Full-length CDH17 cDNA (NM\_004063.4) was obtained from OriGene Technologies, Inc. (Rockville, MD, USA). The CDH17 cDNA was cloned into the pCMV6 vector. The vector was transfected into LN229 or CHO-K1 cells using the Neon transfection system (Thermo Fisher Scientific, Inc.). Stable transfectants were sorted using an anti-CDH17 mAb (clone CDH17/2618, Novus Biologicals, Centennial, CO, USA). Finally, LN229/CDH17 and CHO/CDH17 were established. Type I cadherin-overexpressed CHO-K1: CHO/CDH1, CHO/PA16-CDH2 (CHO/CDH2), CHO/CDH3, CHO/PA16-CDH4 (CHO/CDH4), and CHO/PA16-CDH15 (CHO/CDH15) were previously established [23]. Type II cadherin-overexpressed CHO-K1: CHO/PA16-CDH5 (CHO/CDH5), CHO/CDH6, CHO/PA16-CDH7 (CHO/CDH7), CHO/PA16-CDH8 (CHO/CDH8), CHO/PA16-CDH9 (CHO/CDH9), CHO/PA16-CDH10 (CHO/CDH10), CHO/PA16-CDH11 (CHO/CDH11), CHO/PA16-CDH12 (CHO/CDH12), CHO/PA16-CDH18 (CHO/CDH18), CHO/PA16-CDH19 (CHO/CDH19), CHO/PA16-CDH20 (CHO/CDH20), CHO/PA16-CDH22 (CHO/CDH22), and CHO/PA16-CDH24 (CHO/CDH24) were established previously [27]. A truncated cadherin-overexpressed CHO-K1: CHO/PA16-CDH13 (CHO/CDH13), another 7D cadherin-overexpressed CHO-K1: CHO/PA16-CDH16 (CHO/CDH16), and an atypical cadherin-overexpressed CHO-K1: CHO/PA16-CDH26 (CHO/CDH26) were previously established [27].

Each cadherin expression was confirmed using an anti-CDH1 mAb (clone 67A4, BD Biosciences, Franklin Lakes, NJ, USA), an anti-CDH3 mAb (clone MM0508-9V11, Abcam, Cambridge, UK), an anti-CDH6 mAb (clone 427909, R&D Systems Inc., Minneapolis, MN, USA), an anti-CDH13 mAb (clone 392411, R&D Systems Inc.), an anti-CDH17 mAb (clone CDH17/2618), and anti-PA16-tag mAb (clone NZ-33 [28]) to detect other cadherins.

### 2.3. Hybridoma Production

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The experiment was approved by the Animal Care and Use Committee of Tohoku University (Permit No. 2022MdA-001). A 6-week-old female BALB/cAJcl mouse obtained from CLEA Japan (Tokyo, Japan) was immunized intraperitoneally with LN229/CDH17 ( $1 \times 10^8$  cells). Prior to immunization, LN229/CDH17 cells were collected following brief treatment with 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). For the initial immunization, 2% Alhydrogel adjuvant (InvivoGen, San Diego, CA, USA) was included. This was followed by three weekly intraperitoneal injections of LN229/CDH17 ( $1 \times 10^8$  cells) without adjuvant. Two days before

splenocyte collection, a final intraperitoneal booster injection of LN229/CDH17 ( $1 \times 10^8$  cells) was performed. Splenocytes isolated from an LN229/CDH17-immunized mouse were fused with P3U1 cells using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN, USA). The resulting hybridomas were maintained in the RPMI-1640 culture medium supplemented with hypoxanthine, aminopterin, and thymidine (HAT; Thermo Fisher Scientific, Inc.), 5% BriClone (NICB, Dublin, Ireland), and 5  $\mu\text{g}/\text{mL}$  Plasmocin (InvivoGen). Hybridoma supernatants were screened by flow cytometry using CHO/CDH17 and parental CHO-K1 cells. The hybridoma culture supernatant containing Ca17Mab-5 in serum-free Hybridoma-SFM medium (Thermo Fisher Scientific, Inc.) was subsequently filtered and purified using Ab-Catcher Extra (ProteNova, Kagawa, Japan).

#### 2.4. Flow Cytometry

Cells were harvested with 1 mM EDTA and washed with blocking buffer [0.1% bovine serum albumin in phosphate-buffered saline (PBS)]. The cells were incubated with primary mAbs for 30 min at 4 °C. The cells were then stained with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA, USA). Flow cytometric data were acquired on an SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan) by collecting 5,000 events. Cells were gated on forward scatter (FSC) and side scatter (SSC), and fluorescence intensity was analyzed using FlowJo software (BD Biosciences).

#### 2.5. Calculation of the Binding Affinity by Flow Cytometry

Cells were treated with serial dilutions of Ca17Mab-5 or CDH17/2618. The cells were stained with Alexa Fluor 488-conjugated anti-mouse IgG (1:200 dilution). The data (10,000 events) were collected with the SA3800 Cell Analyzer, and the geometric mean (GeoMean) was calculated in FlowJo. The fitting binding isotherms (vertical axis, GeoMean; horizontal axis, mAb concentration) determined the dissociation constant ( $K_D$ ) values to built-in one-side binding models of GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

#### 2.6. Western Blotting

Cell lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.). Proteins (10  $\mu\text{g}/\text{lane}$ ) were electrophoresed on 5–20% polyacrylamide gels (Wako Pure Chemical Corporation) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% non-fat milk (Nacalai Tesque, Inc.), PVDF membranes were incubated with 1  $\mu\text{g}/\text{mL}$  of Ca17Mab-5, 1  $\mu\text{g}/\text{mL}$  of CDH17/2618, and 2  $\mu\text{g}/\text{mL}$  of an anti-isocitrate dehydrogenase 1 (IDH1) mAb (clone RcMab-1-mG1), followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:1000; Agilent Technologies Inc., Santa Clara, CA, USA). Chemiluminescence signals were developed using Pierce™ ECL Plus (Thermo Fisher Scientific, Inc.) or ImmunoStar LD (Wako Pure Chemical Corporation). The signals were imaged with ChemiDoc Touch MP (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

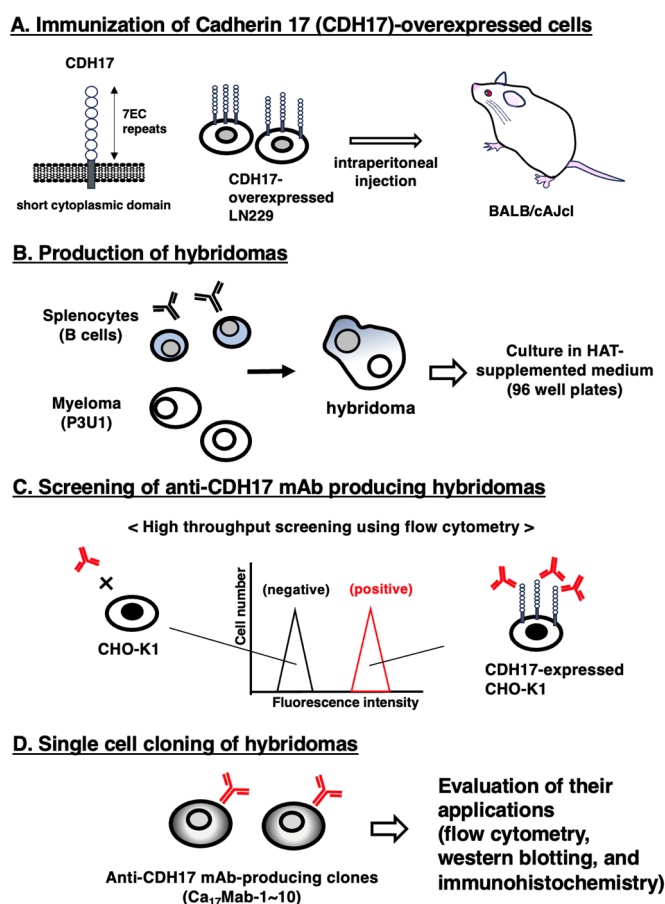
#### 2.7. Immunohistochemistry Using Cell Blocks and Tissue Microarrays

All IHC procedures were performed using the VENTANA BenchMark ULTRA PLUS (Roche Diagnostics, Indianapolis, IN, USA). Cells were fixed with 4% paraformaldehyde, and cell blocks were prepared using iPGell (Genostaff Co., Ltd., Tokyo, Japan). Formalin-fixed, paraffin-embedded (FFPE) cell sections were stained with Ca17Mab-5 (0.2 or 2  $\mu\text{g}/\text{mL}$ ) or CvMab-62 (2  $\mu\text{g}/\text{mL}$ , IgG<sub>1</sub> isotype control). Colorectal cancer microarrays (CO243b, CO352a, CO353a, CO483b, and CO484b, US Biomax Inc., Rockville, MD, USA) were stained with Ca17Mab-5 (5  $\mu\text{g}/\text{mL}$  or 10  $\mu\text{g}/\text{mL}$  in CO243b). Staining was performed on the VENTANA BenchMark ULTRA PLUS using the ultraView Universal DAB Detection Kit (Roche Diagnostics).

### 3. Results

#### 3.1. Development of Anti-CDH17 mAbs by the CBIS Method

We first prepared the immunogen LN229/CDH17 as described in Section 2.2 and Figure 1A. LN229/CDH17 ( $1 \times 10^8$  cells) were injected intraperitoneally five times into a BALB/cAJcl mouse (Figure 1A). Splenocytes were fused with myeloma P3U1 cells to generate hybridomas, which were plated in 96-well plates (Figure 1B). Hybridoma supernatants were screened to identify those positive for CHO/CDH17 and negative for CHO-K1 (Figure 1C). Flow cytometric screening identified 129 of 474 wells (27%) that showed strong signals with CHO/CDH17 cells compared with CHO-K1 cells. Limiting dilution was then performed, and a total of 10 clones producing anti-CDH17 mAbs were established. Supernatants were further evaluated for use in flow cytometry, Western blotting, and immunohistochemistry (Figure 1D). Finally, clone Ca<sub>17</sub>Mab-5 (IgG<sub>1</sub>, κ) was selected because it can be used in all applications ([https://www.med-tohoku-antibody.com/topics/antibody\\_bank.htm](https://www.med-tohoku-antibody.com/topics/antibody_bank.htm), accessed on 18 May 2026).

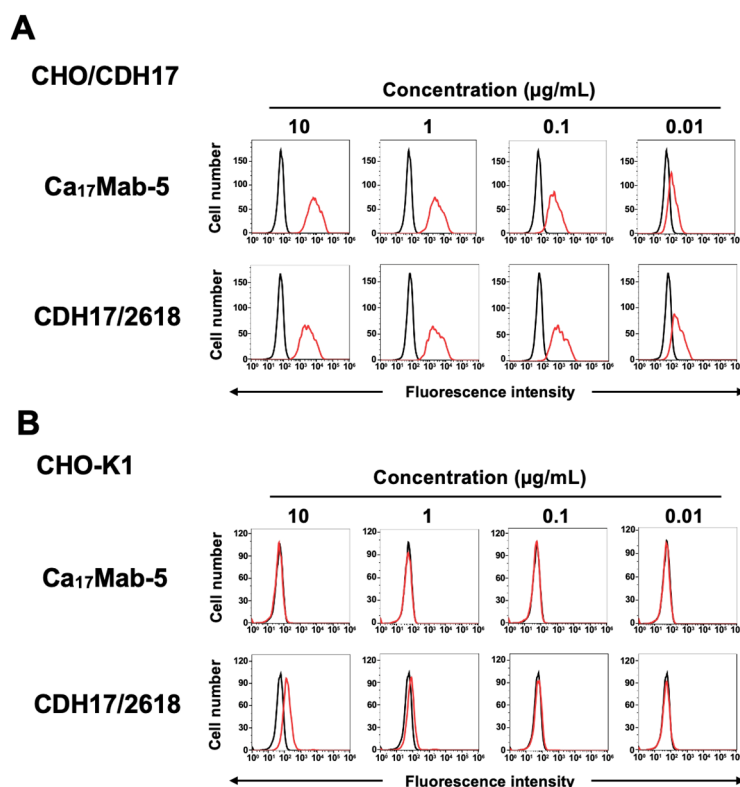


**Figure 1.** Screening of anti-CDH17 mAbs. (A) CDH17-overexpressed LN229 cells (LN229/CDH17) were used to immunize a BALB/cAJcl mouse. (B) After five immunizations, splenocytes were fused with P3U1. (C) Hybridoma culture supernatants were screened by flow cytometry using CHO-K1 and CHO/CDH17. (D) Anti-CDH17 mAb-producing hybridoma clones (Ca<sub>17</sub>Mabs) were established by limiting dilution.

#### 3.2. Flow Cytometric Analysis of Ca<sub>17</sub>Mab-5 Against CHO/CDH17 and CHO-K1

Purified Ca<sub>17</sub>Mab-5 was prepared, and its reactivity was assessed by flow cytometry. As shown in Figure 2A, Ca<sub>17</sub>Mab-5 reacted with CHO/CDH17 in a dose-dependent manner from 10 to 0.01 μg/mL. In contrast, Ca<sub>17</sub>Mab-5 did not recognize parental CHO-K1 even at 10 μg/mL (Figure 2B). Although a commercially available anti-CDH17 mAb (clone CDH17/2618) reacted with CHO/CDH17

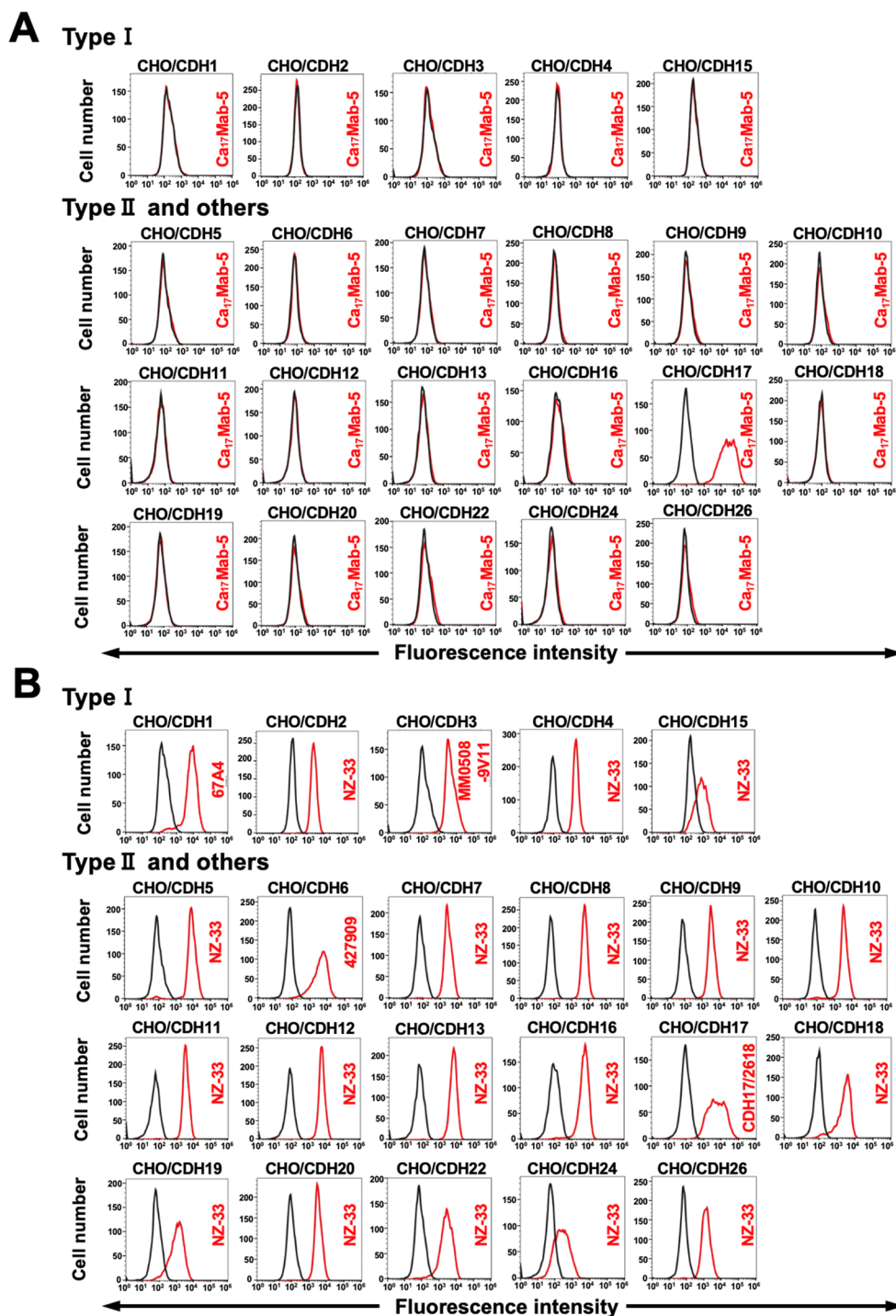
in a dose-dependent manner from 10 to 0.01  $\mu\text{g/mL}$  (Figure 2A), it also non-specifically recognized CHO-K1 at 10  $\mu\text{g/mL}$  (Figure 2B).



**Figure 2.** Flow cytometric analysis using Ca<sub>17</sub>Mab-5 and CDH17/2618. CHO/CDH17 (**A**) and CHO-K1 (**B**) were treated with Ca<sub>17</sub>Mab-5 or CDH17/2618 at the indicated concentrations (red line). The black line represents negative control (blocking buffer). These cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer.

### 3.3. Determination of the Specificity of Ca<sub>17</sub>Mab-5 Using CDHs-Overexpressed CHO-K1

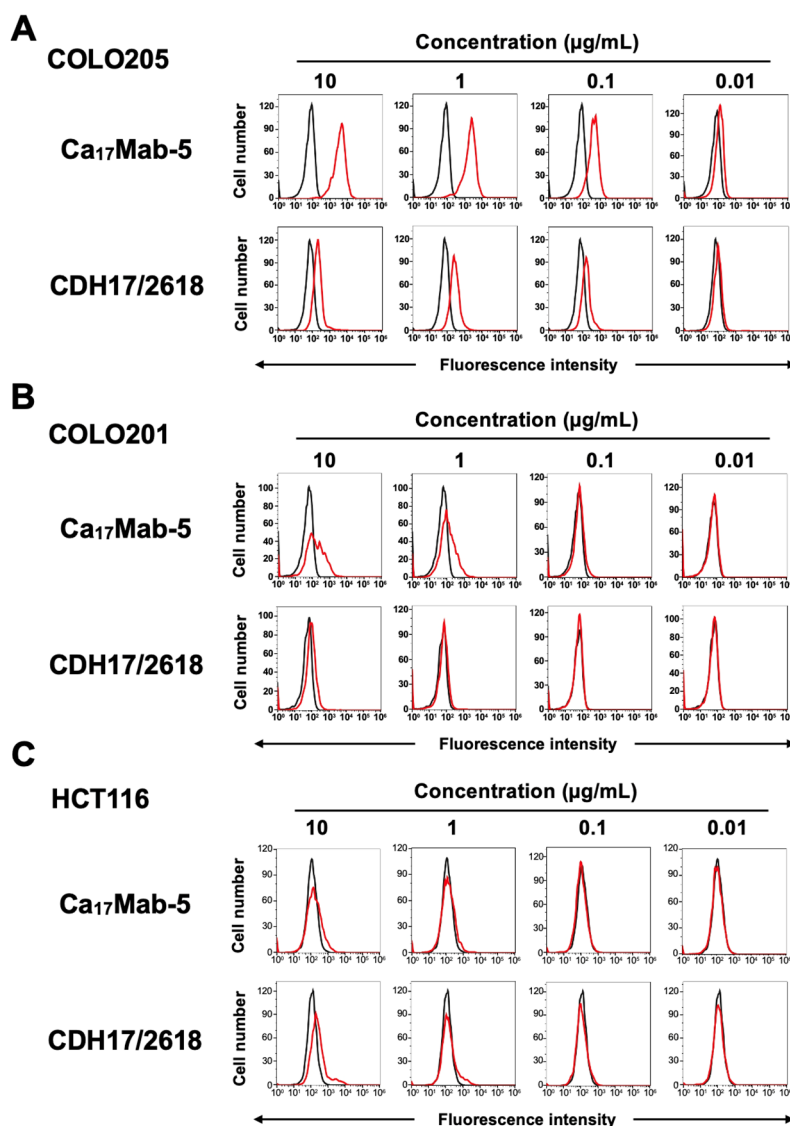
We previously generated CHO-K1 cells which overexpressed type I CDHs (CHO/CDH1, CHO/CDH2, CHO/CDH3, CHO/CDH4, and CHO/CDH15) [23,26], type II CDHs (CHO/CDH5, CHO/CDH6, CHO/CDH7, CHO/CDH8, CHO/CDH9, CHO/CDH10, CHO/CDH11, CHO/CDH12, CHO/CDH18, CHO/CDH19, CHO/CDH20, CHO/CDH22, and CHO/CDH24), another 7D CDH (CHO/CDH16), and an atypical CDH (CHO/CDH26) [27]. Therefore, the specificity of Ca<sub>17</sub>Mab-5 to those CDHs was investigated. As shown in Figure 3A, Ca<sub>17</sub>Mab-5 recognized CHO/CDH17 but did not cross-react with other CDHs-overexpressed CHO-K1. The cell surface expression of each CDH was confirmed in Figure 3B. These results indicate that Ca<sub>17</sub>Mab-5 is a specific mAb to CDH17 among those CDHs.



**Figure 3.** Specificity of Ca<sub>17</sub>Mab-5. (A) The type I CDHs (CDH1, CDH2, CDH3, CDH4, and CDH15), type II CDHs (CDH5, CDH6, CDH7, CDH8, CDH9, CDH10, CDH11, CDH12, CDH18, CDH20, CDH22, and CDH24), a truncated CDH (CDH13), 7D CDHs (CDH16 and CDH17), and an atypical CDH (CDH26)-overexpressed CHO-K1 were treated with 10 µg/mL of Ca<sub>17</sub>Mab-5 (red) or with control blocking buffer (black, negative control), followed by treatment with anti-mouse IgG conjugated with Alexa Fluor 488. (B) Each cadherin expression was confirmed by 10 µg/mL of an anti-CDH1 mAb (clone 67A4), 10 µg/mL of an anti-CDH3 mAb (clone MM0508-9V11), 1 µg/mL of an anti-CDH6 mAb (clone 427909), 1 µg/mL of an anti-CDH17 mAb (clone CDH17/2618), and 0.1 µg/mL of an anti-PA16-tag mAb (clone NZ-33) to detect other CDHs, followed by the treatment with Alexa Fluor 488-conjugated secondary mAbs. The fluorescence data were collected using the SA3800 Cell Analyzer.

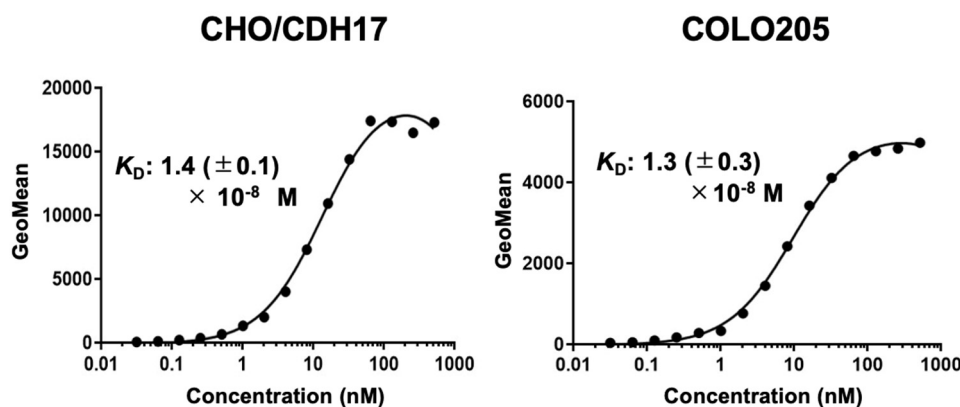
### 3.4. Flow Cytometric Analysis of Ca<sub>17</sub>Mab-5 and CDH17/2618 Against Endogenous CDH17-Positive and Negative Cells

We then searched the expression of CDH17 in various colorectal cancer cell lines using Ca<sub>17</sub>Mab-5 and CDH17/2618. As shown in Figure 4, Ca<sub>17</sub>Mab-5 dose-dependently recognized endogenous CDH17 in COLO205. In contrast, the reactivity of CDH17/2618 was lower than that of Ca<sub>17</sub>Mab-5 (Figure 4A). Ca<sub>17</sub>Mab-5 exhibited a weak reactivity to COLO201, whereas CDH17/2618 barely reacted with COLO201 (Figure 4B). Additionally, Ca<sub>17</sub>Mab-5 and CDH17/2618 hardly recognized HCT116 (Figure 4C). These results indicate that both Ca<sub>17</sub>Mab-5 showed a superior reactivity to endogenous CDH17 compared to CDH17/2618 in flow cytometry.



**Figure 4.** Flow cytometric analysis using Ca<sub>17</sub>Mab-5 and CDH17/2618 to detect endogenous CDH17. COLO205 (A), COLO201 (B), and HCT116 (C) were treated with Ca<sub>17</sub>Mab-5 and CDH17/2618 at the indicated concentrations (red) or blocking buffer (black, negative control). These cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer.

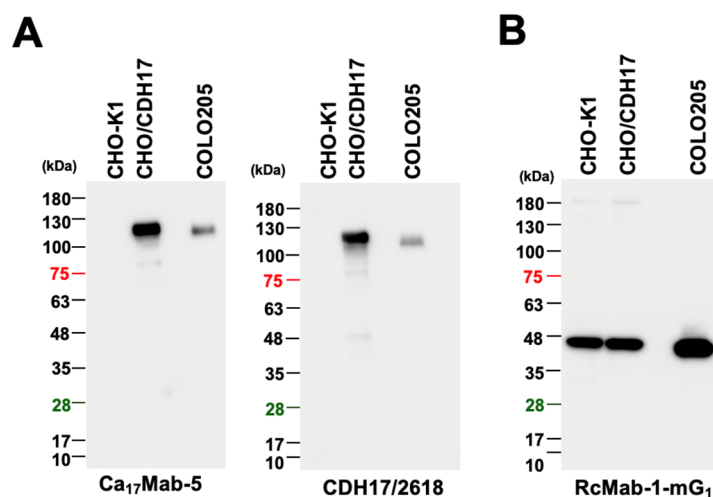
The binding affinity of Ca<sub>17</sub>Mab-5 was measured with CHO/CDH17 and COLO205 using flow cytometry. The  $K_D$  values for Ca<sub>17</sub>Mab-5 with CHO/CDH17 and COLO205 were  $1.4 (\pm 0.1) \times 10^{-8}$  M and  $1.3 (\pm 0.3) \times 10^{-8}$  M, respectively (Figure 5). The  $K_D$  value of CDH17/2618 for COLO205 could not be determined because the sigmoid curve did not reach a plateau (Supplementary Figure 1). These results show that Ca<sub>17</sub>Mab-5 has moderate binding affinity for exogenous and endogenous CDH17.



**Figure 5.** Determination of the binding affinity of Ca<sub>17</sub>Mab-5 by flow cytometry. CHO/CDH17 and COLO205 cells were incubated with serially diluted Ca<sub>17</sub>Mab-5, then reacted with Alexa Fluor 488-conjugated anti-mouse IgG. Geometric mean fluorescence values were measured using the SA3800 Cell Analyzer and FlowJo software. Average  $K_D$  values ( $\pm$  standard deviation) from three independent measurements were calculated using GraphPad PRISM 6. Representative graphs are shown.

### 3.5. Detection of Exogenous and Endogenous CDH17 by Ca<sub>17</sub>Mab-5 and CDH17/2618 in Western Blotting

We next tested whether Ca<sub>17</sub>Mab-5 and CDH17/2618 can be used in Western blotting. As shown in Figure 6A, Ca<sub>17</sub>Mab-5 and CDH17/2618 detected a band of approximately 120 kDa in CHO/CDH17 cell lysates, whereas no band was detected in parental CHO-K1 cell lysates. Additionally, Ca<sub>17</sub>Mab-5 and CDH17/2618 detected endogenous CDH17 in COLO205 cell lysates at 120 kDa (Figure 6A). An anti-IDH1 mAb (clone RcMab-1-mG<sub>1</sub>) served as an internal control (Figure 6B). These results demonstrate that Ca<sub>17</sub>Mab-5 and CDH17/2618 can detect CDH17 by Western blotting.

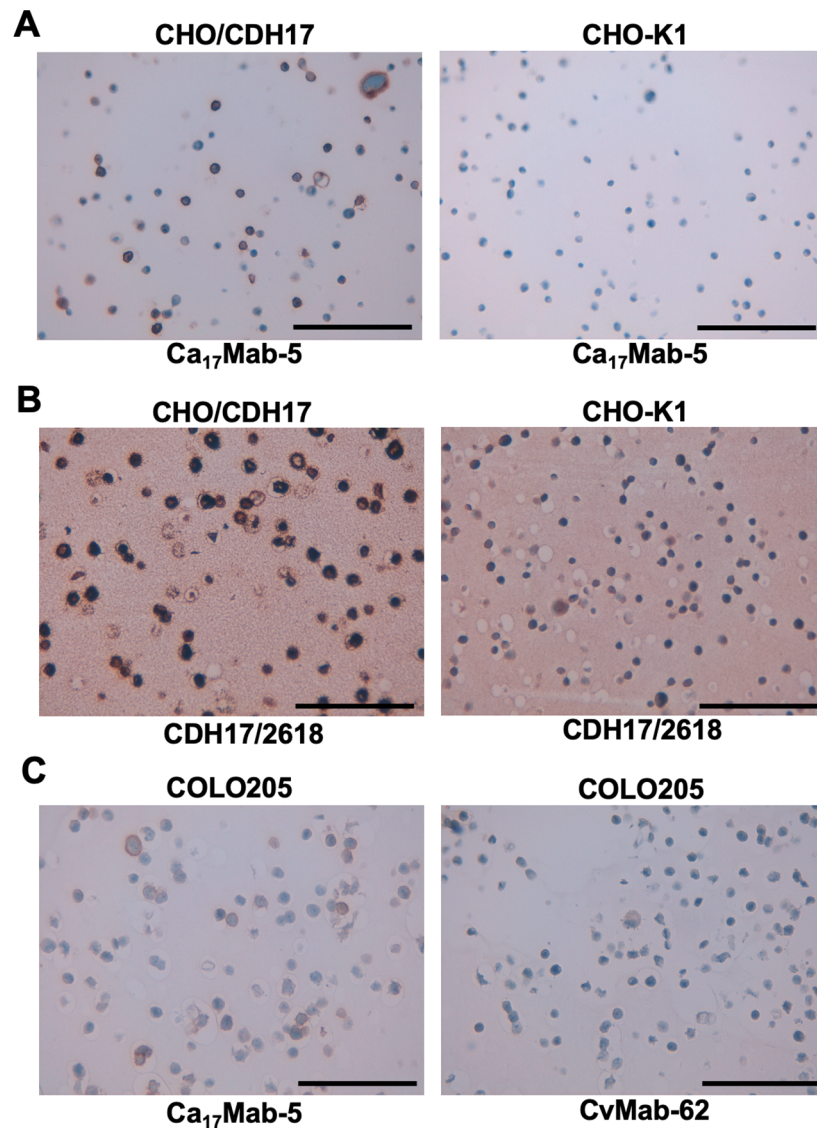


**Figure 6.** Western blotting using Ca<sub>17</sub>Mab-5 and CDH17/2618. Cell lysates (10  $\mu$ g/lane) from CHO-K1, CHO/CDH17, and COLO205 were electrophoresed and transferred to polyvinylidene difluoride membranes. (A) The membranes were incubated with 1  $\mu$ g/mL of Ca<sub>17</sub>Mab-5 or 1  $\mu$ g/mL of CDH17/2618. The detection conditions were the same. (B) An anti-IDH1 mAb (clone RcMab-1-mG<sub>1</sub>, 2  $\mu$ g/mL) was used as an internal control. The membranes were further treated with anti-mouse IgG-conjugated with horseradish peroxidase.

### 3.6. Immunohistochemistry Using Ca<sub>17</sub>Mab-5 in Formalin-Fixed Paraffin-Embedded Cell Blocks and Tissue Microarrays

We tested whether Ca<sub>17</sub>Mab-5 and CDH17/2618 are suitable for IHC in FFPE sections from CHO-K1 and CHO/CDH17. Ca<sub>17</sub>Mab-5 showed clear membranous staining in CHO/CDH17 but not in

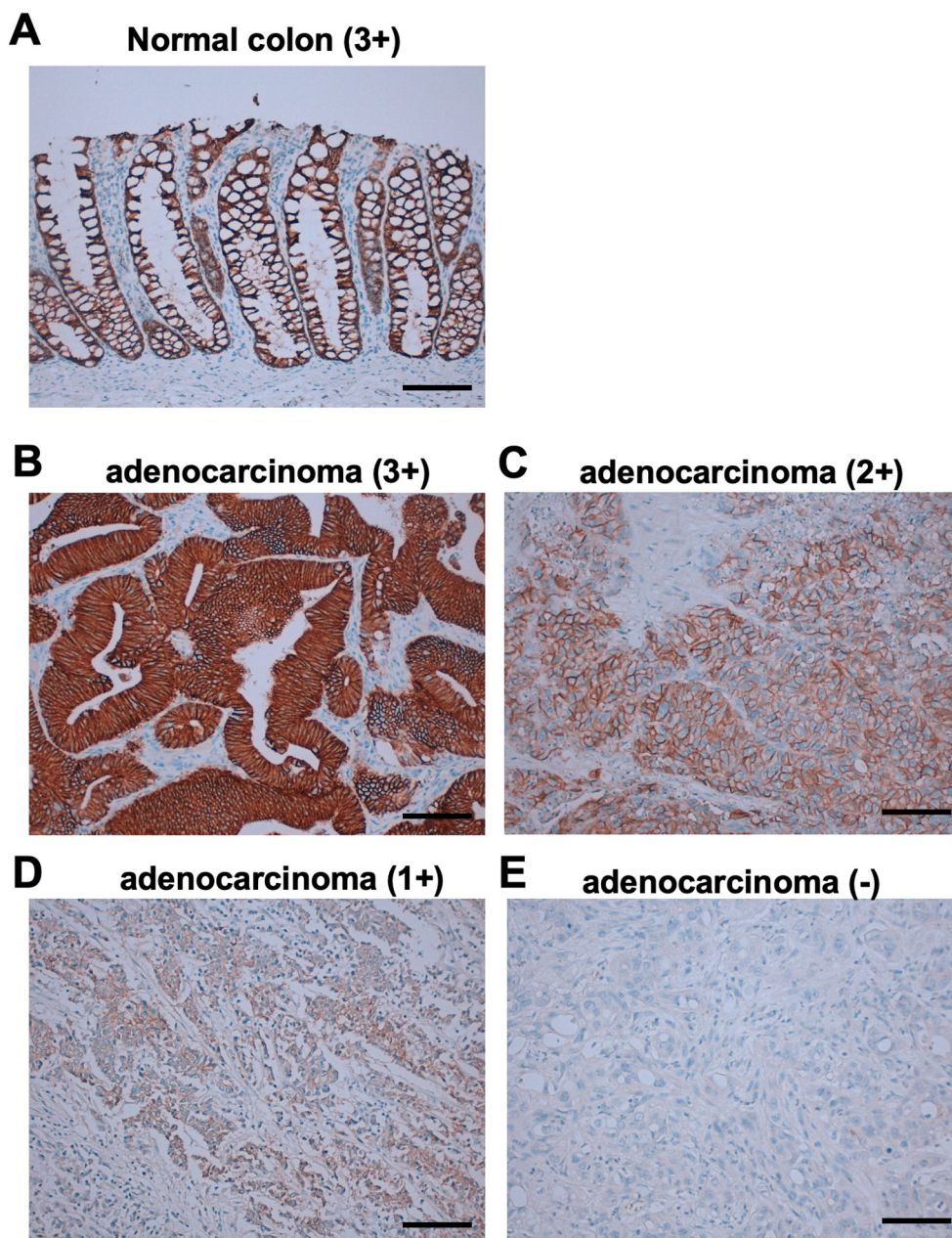
CHO-K1 (Figure 7A). In same experimental setting, CDH17/2618 showed potent reactivity to CHO/CDH17, but weak reactivity to CHO-K1 was also detected (Figure 7B). Furthermore, Ca<sub>17</sub>Mab-5 also displayed membranous staining in COLO205, but the isotype control mAb (CvMab-62) did not (Figure 7C). These results indicate that Ca<sub>17</sub>Mab-5 can detect both exogenous and endogenous CDH17 in IHC of FFPE sections from cultured cells.



**Figure 7.** Immunohistochemistry using Ca<sub>17</sub>Mab-5 and CDH17/2618 in formalin-fixed paraffin-embedded cell blocks. (A) CHO/CDH17 and CHO-K1 sections were treated with 0.2 µg/mL of Ca<sub>17</sub>Mab-5. (B) CHO/CDH17 and CHO-K1 sections were treated with 0.2 µg/mL of CDH17/2618. (C) COLO205 sections were treated with 2 µg/mL of Ca<sub>17</sub>Mab-5 or 2 µg/mL of CvMab-62 (IgG<sub>1</sub> isotype control). The staining was performed using VENTANA BenchMark ULTRA PLUS with the ultraView Universal DAB Detection Kit. Scale bar = 100 µm.

We next stained colorectal cancer tissue microarrays. As shown in Figure 8A, Ca<sub>17</sub>Mab-5 predominantly localized to the basolateral membrane and exhibited strong staining (3+) in normal colon epithelium. In colorectal adenocarcinomas, Ca<sub>17</sub>Mab-5 showed potent membranous staining (3+) in well-differentiated adenocarcinomas (Figure 8B). The abundance of CDH17 detected by Ca<sub>17</sub>Mab-5 tended to be reduced in poorly-differentiated adenocarcinomas [Figure 8C (moderate intensity, 2+) and D (weak intensity, 1+)]. Adenocarcinomas with no CDH17 expression were also observed (Figure 8E). Table 1 summarized the result of colorectal cancer tissue microarray (CO483b), which includes Figure 8B to E. Supplementary Table S1 summarized the result of other colorectal

cancer tissue microarrays. Consequently, Ca<sub>17</sub>Mab-5 stained 133 out of 154 cases (86 %, more than +1). These results indicated that Ca<sub>17</sub>Mab-5 is suitable to detect CDH17 in IHC.



**Figure 8.** Immunohistochemistry using Ca<sub>17</sub>Mab-5 in colorectal cancer tissue arrays. The colorectal cancer tissue arrays were treated with 5 µg/mL of Ca<sub>17</sub>Mab-5. (A) Normal colon epithelium (strong intensity, 3+). The representative images of colorectal adenocarcinoma with strong intensity (3+, B), moderate intensity (2+, C), weak intensity (1+, D), and no staining (-, E) were shown. The staining was performed using VENTANA BenchMark ULTRA PLUS with the ultraView Universal DAB Detection Kit. Scale bar = 100 µm.

**Table 1.** Immunohistochemistry of a colorectal cancer tissue microarray (CO483b) by Ca<sub>17</sub>Mab-5.

No.	Age	Sex	Pathology diagnosis	TNM	Ca <sub>17</sub> Mab-5
1	67	M	Adenocarcinoma	T2N0M0	3+
2	67	M	Adenocarcinoma	T3N1M0	3+
3	48	M	Adenocarcinoma	T3N0M0	3+
4	58	M	Adenocarcinoma	T3N1M0	3+

5	75	M	Adenocarcinoma with necrosis	T3N0M0	3+
6	86	M	Adenocarcinoma	T4N1M0	3+
7	55	M	Adenocarcinoma	T2N0M0	3+
8	38	M	Adenocarcinoma	T4N1M0	3+
9	52	M	Adenocarcinoma	T3N1M0	3+
10	46	M	Mucinous adenocarcinoma	T3N2M0	3+
11	61	M	Mucinous adenocarcinoma (Figure 8B)	T3N0M0	3+
12	55	M	Adenocarcinoma	T3N2M0	2+
13	46	F	Adenocarcinoma	T3N1M0	3+
14	44	M	Adenocarcinoma	T4N1M0	2+
15	31	M	Adenocarcinoma	T4N1M0	2+
16	74	F	Adenocarcinoma	T4N0M0	3+
17	61	M	Adenocarcinoma (Figure 8E)	T3N0M0	-
18	45	M	Adenocarcinoma	T4N1M0	1+
19	58	M	Mucinous adenocarcinoma	T3N0M0	2+
20	78	M	Adenocarcinoma with necrosis	T3N1M1	3+
21	69	M	Adenocarcinoma	T4N2M0	2+
22	64	F	Adenocarcinoma (Figure 8C)	T4N1M0	2+
23	82	M	Mucinous adenocarcinoma	T4N0M0	2+
24	34	M	Adenocarcinoma (Figure 8D)	T3N0M0	1+
25	50	F	Adenocarcinoma	T4N0M0	2+
26	34	F	Adenocarcinoma	T3N0M0	1+
27	52	F	Adenocarcinoma	T3N2M0	2+
28	53	F	Adenocarcinoma	T2N0M0	2+
29	58	F	Adenocarcinoma	T3N0M0	-
30	59	F	Adenocarcinoma	T3N1M0	1+
31	67	M	Adenocarcinoma	T3N2M0	1+
32	31	M	Adenocarcinoma	T4N0M0	-
33	54	F	Adenocarcinoma	T4N1M0	-
34	62	M	Adenocarcinoma	T2N0M0	1+
35	80	M	Adenocarcinoma	T4N2M0	2+
36	67	F	Adenocarcinoma	T3N0M0	1+
37	52	F	Adenocarcinoma	T3N1M0	1+
38	73	M	Adenocarcinoma	T3N0M0	-
39	75	M	Adenocarcinoma	T4N0M0	-
40	57	F	Adenocarcinoma	T2N0M0	2+
41	30	M	Sigmoid colon tissue	normal	2+
42	22	M	Sigmoid colon tissue	normal	3+
43	30	M	Colon tissue	normal	3+
44	35	M	Colon tissue	normal	3+
45	45	M	Colon tissue	normal	3+

46	32	M	Ascending colon tissue	normal	3+
47	32	M	Colon tissue	normal	3+

–, No stain; 1+, Weak intensity; 2+, Moderate intensity; 3+, Strong intensity.

#### 4. Discussion

CDH17 is expressed almost exclusively in intestinal epithelial cells of the embryonic and adult small intestine and colon [29]. Consistent with this restricted tissue distribution, CDH17 is regarded as a selective marker of colon cancer cell lines and is expressed in 35 out of 54 epithelial-like colon cancer cell lines included in the Cancer Cell Line Encyclopedia [30]. In this study, we developed novel anti-CDH17 mAbs by immunizing a mouse with CDH17-overexpressed LN229 cells (Figure 1). A clone Ca<sub>17</sub>Mab-5 recognized both exogenous and endogenous CDH17 in flow cytometry (Figures 2 and 4) and IHC (Figures 7 and 8). Ca<sub>17</sub>Mab-5 was able to detect CDH17 in COLO205 but not HCT116 (Figure 4), which is consistent with the gene expression profiles of colorectal cancer cell lines in the NCI-60 human cancer cell line panel [30]. Importantly, Ca<sub>17</sub>Mab-5 specifically recognized CDH17 without detectable cross-reactivity to other 21 cadherins including classical type I and type II CDHs, and other types of CDHs (Figure 3). Therefore, Ca<sub>17</sub>Mab-5 will be useful for the specific isolation of CDH17-positive cells via fluorescence-activated cell sorting for basic research.

A commercially available anti-CDH17 mAb (clone CDH17/2618) was developed by immunization of recombinant fragment (amino acids 242-418) of human CDH17 ([https://www.novusbio.com/products/cadherin-17-antibody-cdh17-2618\\_nbp2-79723?srsid=AfmBOoqdTWDqYHSRLjuVYR-gDiwQheUveNYpDjFFCxpS\\_btPMehtSecp#datasheet](https://www.novusbio.com/products/cadherin-17-antibody-cdh17-2618_nbp2-79723?srsid=AfmBOoqdTWDqYHSRLjuVYR-gDiwQheUveNYpDjFFCxpS_btPMehtSecp#datasheet), accessed on 18 May 2026). CDH17/2618 similarly detected both exogenous and endogenous CDH17 in Western blotting (Figure 6). However, the reactivity to endogenous CDH17 in flow cytometry was low compared to that of Ca<sub>17</sub>Mab-5 (Figure 4A). Weak reactivities that appears to be in the background were observed at 10 µg/mL (Figure 2B and Figure 4). This may be a reason why the sigmoid curve of CDH17/2618 did not reach a plateau (Supplementary Figure S1). The strategy of CDH17/2618 selection was not described in above WEB site. Since Ca<sub>17</sub>Mab-5 was selected by a flow cytometry-based screening (Figure 1C), Ca<sub>17</sub>Mab-5 showed a superior reactivity to COLO205, which is thought to be important to target tumor cells *in vivo* for the development of therapeutic mAb. Furthermore, Ca<sub>17</sub>Mab-5 can be used for IHC of cell specimens (Figure 7) and tissue microarrays (Figure 8). Notably, IHC was conducted by an automated slide-staining system, VENTANA BenchMark ULTRA PLUS, which ensures reproducible staining conditions and accurate assessment of target expression for diagnosis.

CDH17 is aberrantly expressed in not only colorectal cancer [11,31], but also gastric cancer [32,33], ovarian cancer [34], hepatocellular carcinoma [35], and pancreatic neuroendocrine tumor [36]. CDH17 transcription is regulated by CDX2, an intestine-specific caudal-related homeobox transcription factor that plays an important role in the regulation of intestinal epithelium homeostasis [37,38]. CDX2 also mediates gastric intestinal metaplasia [39,40], a precancerous lesion defined by the replacement of gastric mucosa with intestinal-like epithelium, with a considerable risk for gastric cancer without effective therapeutic strategies [41–43]. Functionally, CDH17 has been shown to be a critical regulator of the stemness and chemoresistance through upregulation of LGR5/Wnt/MYC axis and a glutamine transporter SLC38A5, respectively [17]. Anti-CDH17 RGD mAb (clone 6.6.1), an inhibitor of  $\alpha 2\beta 1$  integrin-CDH17 interaction, suppressed LGR5 expression and downstream Wnt signaling activity, suggesting that CDH17- $\alpha 2\beta 1$  integrin interaction promotes the acquisition of stemness in colorectal cancer cells [17,44]. Therefore, it is worthwhile to evaluate the effect of Ca<sub>17</sub>Mab-5 and other Ca<sub>17</sub>Mabs in the maintenance of stemness in the future. Moreover, identification of the Ca<sub>17</sub>Mab epitopes is also essential to investigate the relationship between the neutralization activity and the biological effects.

To target CDH17-positive tumors, multiple therapeutic strategies including mAb monotherapy [44], bispecific Abs such as TRAILR2 × CDH17 [45,46] and CDH3 × CDH17 [47], ADCs [48],

immunotoxins [49], radiolabeled agents [50], CAR-T [51,52], and CAR-NK cell [53] therapies have been developed in preclinical studies. Some of the modalities have been evaluated in clinical trials [54]. We previously cloned mAb cDNAs from hybridomas and generated recombinant mouse IgG<sub>2a</sub> mAbs to enhance antibody-dependent cellular cytotoxicity (ADCC), followed by evaluation of their antitumor efficacy in human tumor xenograft models [55]. We have successfully cloned the cDNA encoding Ca<sub>17</sub>Mab-5, and an isotype-converted mouse IgG<sub>2a</sub>- or human IgG<sub>1</sub>-type Ca<sub>17</sub>Mab-5 will be generated and assessed for its antitumor efficacy using *in vitro* ADCC assays and tumor xenograft models.

As shown in Figure 8A and previous observation, CDH17 is predominantly localized to the basolateral membrane of normal intestinal epithelium [52]. This restricted distribution is thought to minimize ADC or CAR-T cell accessibility in normal tissues, which reduces the risk of on-target, off-tumor toxicities [48,52]. However, there is no preclinical models that accurately predict anti-CDH17 therapy associated toxicities, which raises concerns about on-target, off-tumor toxicities. Adverse effects including diarrhea and mucositis could narrow the therapeutic window for clinical applications.

To achieve a favorable therapeutic window with reducing on-target, off-tumor toxicities, we have developed cancer-specific mAbs (CasMabs) directed several tumor-associated antigens, including podoplanin [56], podocalyxin [57], and human epidermal growth factor receptor 2 (HER2) [58], and successfully identified their corresponding cancer-specific epitopes. Among approximately 300 anti-HER2 mAb clones, the anti-HER2 CasMab, H<sub>2</sub>CasMab-2 was selected based on its cancer-selective binding profile in flow cytometry [58]. H<sub>2</sub>CasMab-2 selectively recognized HER2-expressing breast cancer cells, while exhibiting no detectable reactivity toward normal cells including colon epithelial cells [58]. Additionally, we elucidated the structural basis of the interaction between H<sub>2</sub>CasMab-2 and extracellular domain IV of HER2 [59]. Furthermore, a single-chain variable fragment (scFv) derived from H<sub>2</sub>CasMab-2 was incorporated into chimeric antigen receptor (CAR) T cells, which exhibited cancer-selective reactivity and potent antitumor activity in preclinical models [59]. H<sub>2</sub>CasMab-2-derived CAR-T therapy has been evaluated in a phase I clinical trial for patients with HER2-positive advanced solid tumors (NCT06241456). Therefore, these findings underscore the importance of developing CasMabs against CDH17. Moreover, defining their cancer-specific epitopes is a critical step toward the establishment of safe and effective therapeutic anti-CDH17 CasMabs and related modalities. For this purpose, we are going to increase the clones of Ca<sub>17</sub>Mabs and screen CasMabs against CDH17 in the future.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Determination of binding affinity of CDH17/2618 by flow cytometry. Table S1: Immunohistochemistry of colorectal cancer tissue microarrays by Ca<sub>17</sub>Mab-5.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available in the article.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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