A Novel Anti-Cadherin 19 Monoclonal Antibody (Ca19Mab-8) for Flow Cytometry, Western Blotting, and Immunohistochemistry

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Article

A Novel Anti-Cadherin 19 Monoclonal Antibody (Ca19Mab-8) for Flow Cytometry, Western Blotting, and Immunohistochemistry

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Abstract

A type II cadherin, Cadherin-19 (CDH19), plays a vital role in neural crest development. CDH19 regulates cell–cell junctions and migration by forming catenin-cytoskeleton complexes. Although anti-CDH19 monoclonal antibodies (mAbs) are used for specific applications such as Western blotting and immunohistochemistry (IHC), suitable anti-CDH19 mAbs for flow cytometry are limited. Here, novel anti-human CDH19 mAbs (Ca19Mabs) were developed through flow cytometry-based high-throughput screening. One clone, Ca19Mab-8 (IgG_1, κ), specifically recognized CDH19-overexpressing Chinese hamster ovary-K1 cells but did not bind to other 21 CDHs (including both type I and type II) in flow cytometry. Additionally, Ca19Mab-8 recognized endogenous CDH19 in the human glioblastoma cell line LN229. The dissociation constant (K_D) of Ca19Mab-8 for LN229/CDH19 was 9.0×10^{-9} M. Ca19Mab-8 can detect CDH19 in Western blotting and IHC in human melanoma tissue. These findings suggest that Ca19Mab-8 is versatile for basic research and has potential applications in clinical diagnosis and tumor therapy.

Keywords: Cadherin-19; CDH19; cell-based immunization and screening; monoclonal antibody; flow cytometry; immunohistochemistry

1. Introduction

Cadherin-19 (CDH19) is a neural crest–specific adhesion molecule that plays a central role in maintaining brain homeostasis [1,2]. CDH19 belongs to the classical type II cadherin family and comprises five extracellular cadherin repeats (EC1–EC5), a single-pass transmembrane region, and a cytoplasmic tail [3]. The extracellular domain of CDH19 mediates calcium-dependent homophilic interactions at adherens junctions [4]. Its cytoplasmic domain associates with α-catenin, β-catenin, and p120-catenin, which link CDH19 to the actin cytoskeleton [5,6].

The human CDH19 gene was cloned in 2000 based on its sequence similarity to CDH7 [7]. Expressed sequence tags for CDH19 were isolated from melanocyte cDNA libraries, suggesting that CDH19 expression may be restricted to neural crest-derived cells [7]. Supporting this observation, rat CDH19 was primarily expressed in nerve ganglia and Schwann cells during embryonic development. Moreover, CDH19 expression overlapped with neural crest markers such as AP-2 and Sox10 [2]. In Sox10-knockout mouse embryos, neural crest cells exhibited delayed migration to the distal hindgut along with a simultaneous downregulation of CDH19 [1]. Furthermore, Sox10 was found to bind to the CDH19 promoter, indicating that CDH19 is a direct target of Sox10 during early neural crest cell migration by forming CDH19-catenins-cytoskeleton complexes [1]. Through these functions, CDH19 is crucial for the development of neural crest cells, providing insights into the pathogenesis of nervous system developmental defects [8,9].

Melanoma is a type of skin cancer caused by the oncogenic transformation of melanocytes, which are pigment-producing skin cells derived from the neural crest [10,11]. Estimated new cases

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of melanoma in 2025 are more than 100,000 in the US [12], and approximately 20% of patients have advanced disease [American Joint Committee on Cancer (AJCC) stages: IIIA~IIID] at the time of diagnosis [13]. Although primary melanoma can be cured by surgery, and immune checkpoint inhibitors are the standard care in patients with advanced-stage melanoma [14,15], melanoma is the leading cause of death from skin disease in the US, responsible for 8,430 estimated deaths in 2025 [12]. A single-cell multi-omics analysis of human melanoma revealed that melanoma cells were grouped into seven subtypes, which include a CDH19-high subtype, which was suggested to be more resistant to NK and T cell-mediated immunity [16].

Monoclonal antibodies (mAbs) that detect CDH19 by Western blotting or immunohistochemistry (IHC) have been developed for various applications. However, no mAb is available for flow cytometry. Using the Cell-Based Immunization and Screening (CBIS) method, anti-E-Cadherin/CDH1 [17] and anti-M-Cadherin/CDH15 [18] mAbs for flow cytometry, Western blotting, and IHC were developed by our laboratory. The CBIS method includes high-throughput flow cytometry–based screening. Therefore, mAbs obtained by the CBIS method generally recognize conformational epitopes, enabling their use in flow cytometry. Some of the mAbs are suitable for Western blotting and IHC. In this study, we employed the CBIS method to develop highly versatile anti-CDH19 mAbs.

2. Materials and Methods

2.1. Cell Lines

Chinese hamster ovary (CHO)-K1, mouse myeloma P3X63Ag8U.1 (P3U1), and human glioblastoma (GBM) LN229 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2. Plasmid Construction and Establishment of Stable Transfectants

Genes encoding human *CDH19* (NM_021153) were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The *CDH19* cDNA without the pro-peptide was subcloned into the pCAG-Ble vector with an N-terminal PA16 tag [19]. Additionally, the *CDH19* cDNA with an N-terminal MAP16 tag [20] was constructed. These plasmids were transfected into CHO-K1 or LN229 cells, and stable transfectants were sorted using anti-PA16 tag mAb (clone NZ-1) [19] or anti-MAP16 tag mAb (clone PMab-1) [20] using the Neon transfection system (Thermo Fisher Scientific, Inc.). Finally, PA16-CDH19-overexpressed CHO-K1 (CHO/CDH19) and MAP16-CDH19-overexpressed LN229 (LN229/CDH19) were established.

We previously established other CDH-overexpressed stable transfectants [21]. To confirm the expression of CDHs in these transfectants, 1 μg/mL of an anti-CDH1 mAb (clone 67A4), 1 μg/mL of an anti-CDH3 mAb (clone MM0508-9V11), or 0.1 μg/mL of an anti-PA16 tag mAb, NZ-33 [22] were used.

2.3. Production of Hybridomas

Female BALB/cAJcl mice (CLEA Japan, Tokyo, Japan) were intraperitoneally immunized with LN229/CDH19 cells (1×10^8 cells/injection) mixed with 2% Alhydrogel adjuvant (InvivoGen, San Diego, CA, USA). Following three additional weekly immunizations (1.0×10^8 cells/injection), a booster dose (1×10^8 cells/injection) was administered two days before spleen excision. Hybridomas were produced as previously described [18].

2.4. Flow Cytometry

A total of 1×10^5 cells harvested with 1 mM EDTA were washed with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA; blocking buffer) and incubated with mAbs for 30 minutes at 4 °C. Flow cytometric data were acquired as described previously [17].

 doi:10.20944/preprints202601.0319.v2

2.5. Determination of Dissociation Constant Values Using Flow Cytometry

LN229/CDH19 cells were treated with serially diluted Ca19Mab-8. The dissociation constant (K_D) values were calculated s described previously [17].

2.6. Western Blotting

Western blotting was conducted using 1 μg/mL Ca19Mab-8 or 1 μg/mL of an anti-isocitrate dehydrogenase 1 (IDH1) mAb (clone RcMab-1) as described previously [18].

2.7. IHC Using Cell Blocks and Tissue Arrays

All procedures of IHC were performed using VENTANA BenchMark ULTRA PLUS *(Roche Diagnostics,* Indianapolis, IN, USA*)*. The formalin-fixed paraffin-embedded (FFPE) cell sections were prepared as described previously [18].They are stained with 0.2 μg/mL of Ca19Mab-8 or 0.01 μg/mL of NZ-33 using the *BenchMark* ULTRA *PLUS with* the ultraView Universal DAB Detection Kit *(Roche Diagnostics*, Indianapolis*). A* malignant melanoma (ME241a) and a glioblastoma (GL806e) tissue arrays (US Biomax Inc., Rockville, MD, USA) were stained with 2 μg/mL of Ca19Mab-8 or 2 μg/mL of isotype control IgG1 (CvMab-62).

3. Results

3.1. Anti-CDH19 mAb Development by the CBIS Method

As described in the Materials and Methods, an immunogen, LN229/CDH19, was prepared. LN229/CDH19 (1 × 10^8 cells/mouse) was immunized five times into two BALB/cAJcl mice (Figure 1A). Subsequently, hybridomas were generated by fusing splenocytes with myeloma P3U1 (Figure 1B). The hybridoma supernatants were screened to identify those positive for CHO/CDH19 and negative for CHO-K1 (Figure 1C). As a result, 43 positive wells out of 958 (4.5%) were found. Limiting dilution was then performed to clone hybridomas producing anti-CDH19 mAb (Figure 1D). Ultimately, 12 clones were established (http://www.med-tohoku-antibody.com/topics/001_paper_antibody_PDIS.htm#CDH19+), and the purified mAbs (IgG1 isotype) were prepared.

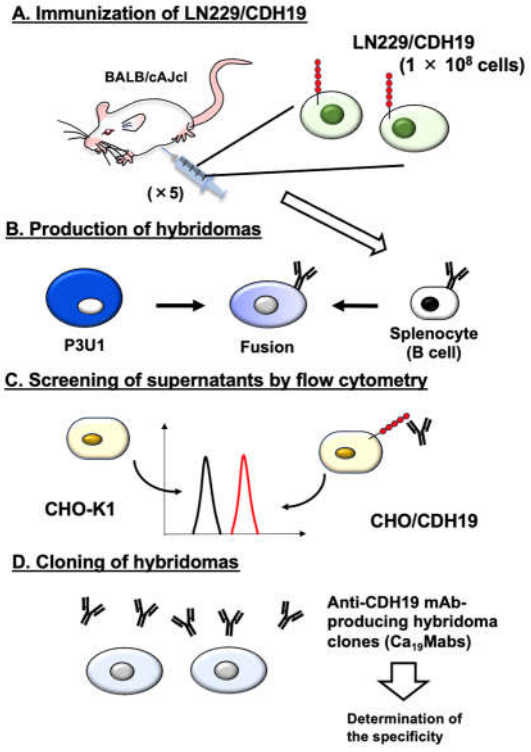

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Figure 1. Schematic depiction of anti-CDH19 mAbs production. A) LN229/CDH19 was injected into BALB/cAJcl mice intraperitoneally. (B) After five immunizations, spleen cells were fused with P3U1. (C) The supernatants from hybridomas were screened by flow cytometry using CHO/CDH19 and CHO-K1 cells. (D) Anti-CDH19 mAb-producing hybridoma clones (Ca19Mabs) were established through limiting dilution.

3.2. Determination of the Specificity of Ca19Mab-8 Using CDHs-Overexpressed CHO-K1

We previously established CHO-K1 cells, which overexpressed type I CDHs (CDH1, CDH2, CDH3, CDH4, and CDH15) [17,18], type II CDHs (CDH5, CDH6, CDH7, CDH8, CDH9, CDH10, CDH11, CDH12, CDH18, CDH19, CDH20, CDH22, and CDH24), 7D CDHs (CDH16 and CDH17), a truncated CDH (CDH13), and an atypical CDH (CDH26) [21]. Therefore, the specificity of Ca19Mabs to those CDHs was determined. As shown in Figure 2A, Ca19Mab-8 reacted with CHO/CDH19 but did not react with other CDHs-overexpressed in CHO-K1. The cell surface expression of CDHs was confirmed in Figure 2B. In contrast, other Ca19Mabs showed cross-reactivity against other type II CDHs (Table 1). These results indicate that Ca19Mab-8 is a specific mAb to CDH19 among those CDHs.

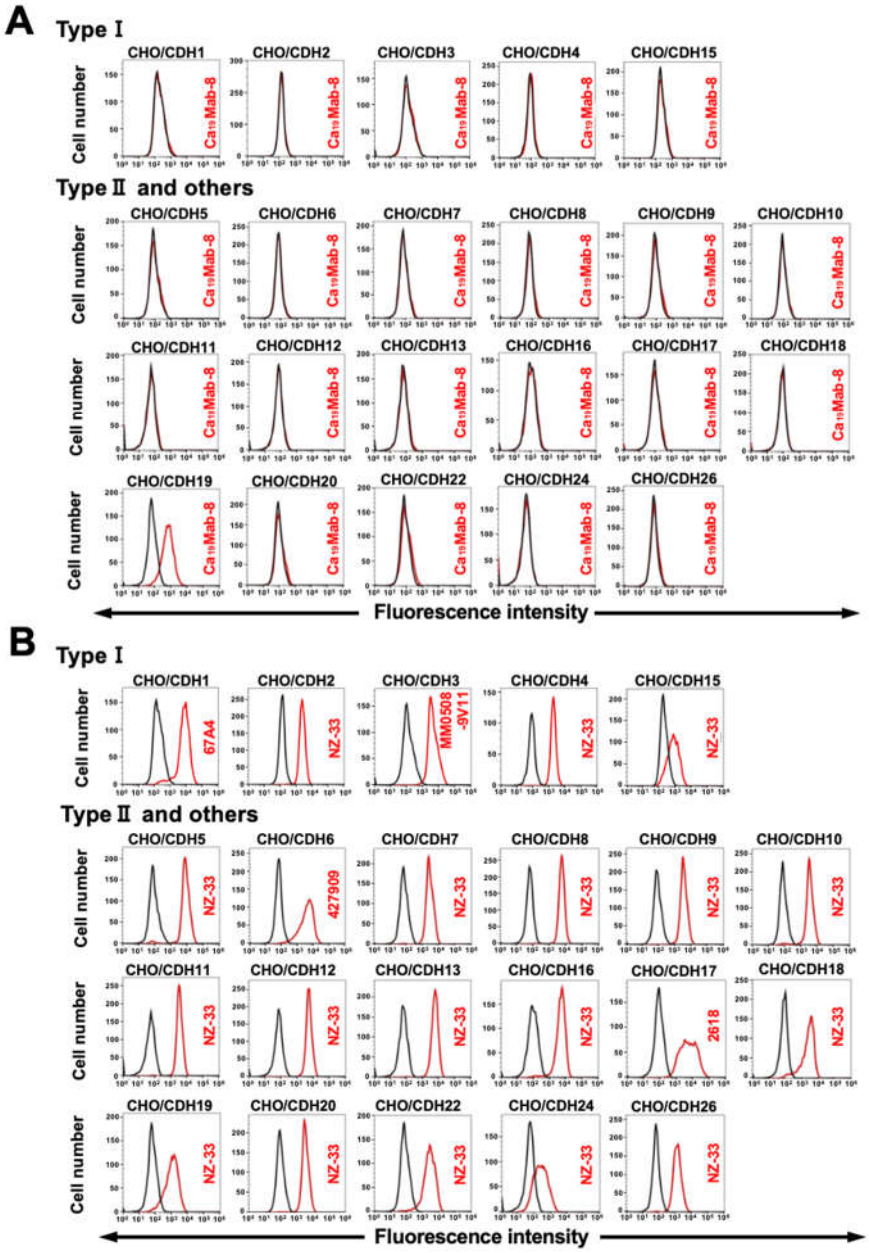

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Figure 2. Flow cytometry analysis of Ca19Mab-8 in CDHs-overexpressed CHO-K1. (A) The type I CDHs (CDH1, CDH2, CDH3, CDH4, and CDH15), type II CDHs (CDH5, CDH6, CDH7, CDH8, CDH9, CDH10, CDH11, CDH12, CDH18, CDH19, CDH20, CDH22, and CDH24), 7D CDHs (CDH16 and CDH17), a truncated CDH (CDH13), and an atypical CDH (CDH26)-overexpressed CHO-K1 were treated with 10 μg/mL of Ca19Mab-8 (red) or with control blocking buffer (black, negative control), followed by treatment with anti-mouse IgG conjugated with Alexa Fluor 488. (B) Each CDH expression was confirmed by 1 μg/mL of an anti-CDH1 mAb (clone 67A4, BD Biosciences), 1 μg/mL of an anti-CDH3 mAb (clone MM0508-9V11, Abcam), and 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.

Table 1. Cross-reactivity of Ca19Mabs (IgG1 isotype) in flow cytometry.

	Isotype	Cross-reactivity
Ca19Mab-1	IgG1, κ	CDH9
Ca19Mab-2	IgG1, κ	CDH6, CDH9, CDH 20
Ca19Mab-3	IgG1, κ	CDH6, CDH9
Ca19Mab-5	IgG1, κ	CDH7, CDH8, CDH11, CDH12, CDH18, CDH20, CDH22, CDH24
Ca19Mab-6	IgG1, κ	CDH7, CDH8, CDH9, CDH11, CDH12, CDH18, CDH20, CDH22 CDH24
Ca19Mab-7	IgG1, κ	CDH7, CDH8, CDH9, CDH10, CDH11, CDH12, CDH18, CDH22
Ca19Mab-8	IgG1, κ	–*1
Ca19Mab-9	IgG1, κ	–*2
Ca19Mab-11	IgG1, κ	CDH8, CDH9, CDH11, CDH12, CDH18, CDH22
Ca19Mab-12	IgG1, κ	CDH9, CDH20

Cross-reactivity was determined by flow cytometry among type II CDHs (CDH5, CDH6, CDH7, CDH8, CDH9, CDH10, CDH11, CDH12, CDH18, CDH19, CDH20, CDH22, and CDH24), 7D CDHs (CDH16 and CDH17), and a truncated CDH (CDH13). *1The data was presented in Figure 2. *2Ca19Mab-9 showed a reactivity to parental CHO-K1.

3.3. Flow Cytometry of Ca19Mab-8 Against CDH19-Overexpressed CHO-K1 and LN229

We next conducted flow cytometry using Ca19Mab-8 (IgG1, κ) against CHO/CDH19, CHO-K1, LN229/CDH19, and LN229. Ca19Mab-8 reacted with CHO/CDH19 in a dose-dependent manner, from 10 to 0.01 μg/mL (Figure 3A). In contrast, Ca19Mab-8 did not recognize CHO-K1 at 10 μg/mL (Figure 3A). Furthermore, Ca19Mab-8 reacted with LN229/CDH19 in a dose-dependent manner (Figure 3B). Ca19Mab-8 also showed reactivity to parental LN229, suggesting that LN229 expressed endogenous CDH19. The binding affinity of Ca19Mab-8 was measured using flow cytometry. The fitting binding isotherms of Ca19Mab-8 to LN229/CDH19 are shown in Figure 3C. The K_D value of Ca19Mab-8 for LN229/CDH19 was 9.0×10^{-9} M. These results indicate that Ca19Mab-8 possesses a moderate binding affinity to LN229/CDH19.

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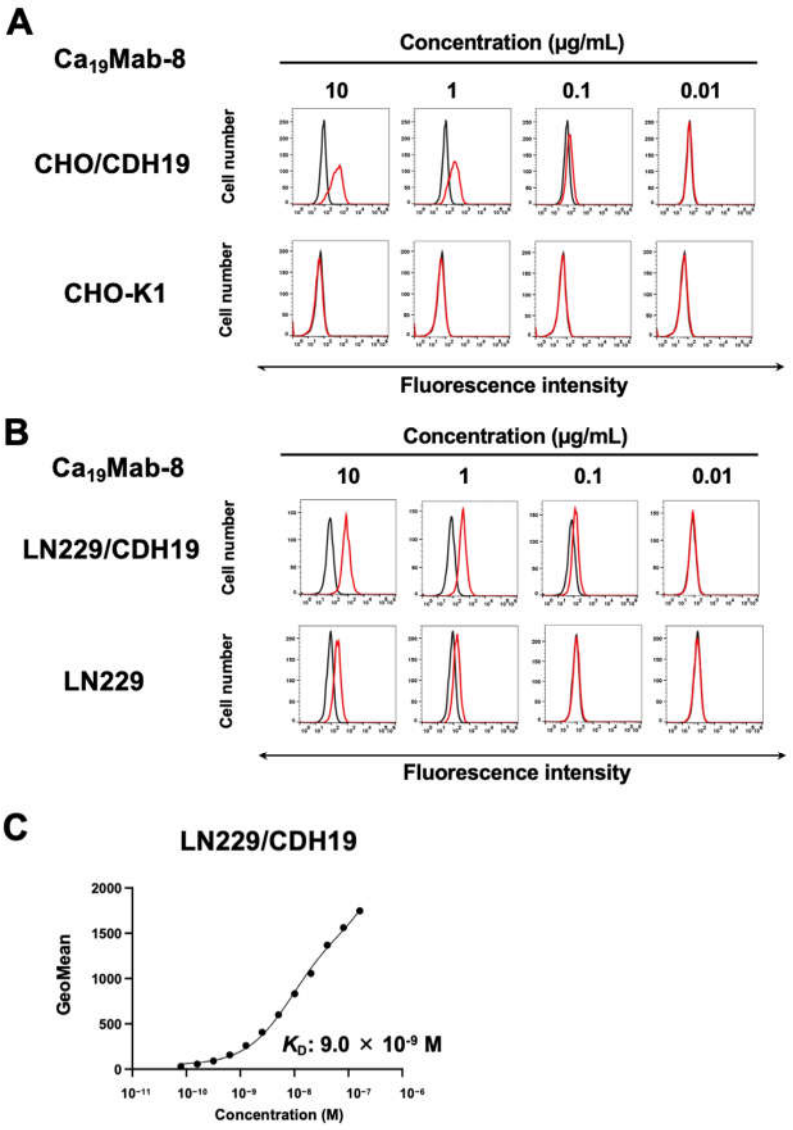

Figure 3. Flow cytometric analysis of Ca19Mab-8. (A) CHO/CDH19 and CHO-K1 were treated with Ca19Mab-8 at the indicated concentrations (red) or with blocking buffer (black, negative control). (B) LN229/CDH19 and LN229 were treated with Ca19Mab-8 at the indicated concentrations (red) or with blocking buffer (black, negative control). The mAbs-treated cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer.

3.4. Western Blotting Using Ca19Mab-8

We then examined whether Ca19Mab-8 is suitable for Western blotting. Whole-cell lysates from CHO-K1, CHO/CDH19, LN229, and LN229/CDH19 were analyzed. Ca19Mab-8 detected clear bands around 90–100 kDa in CHO/CDH19 and LN229/CDH19, but not in CHO-K1 (Figure 4A). It also detected weak bands in parental LN229 (Figure 4A). Figure 4B shows an internal control, IDH1, detected by RcMab-1. These results demonstrate that Ca19Mab-8 can detect both exogenous and endogenous CDH19 in Western blotting.

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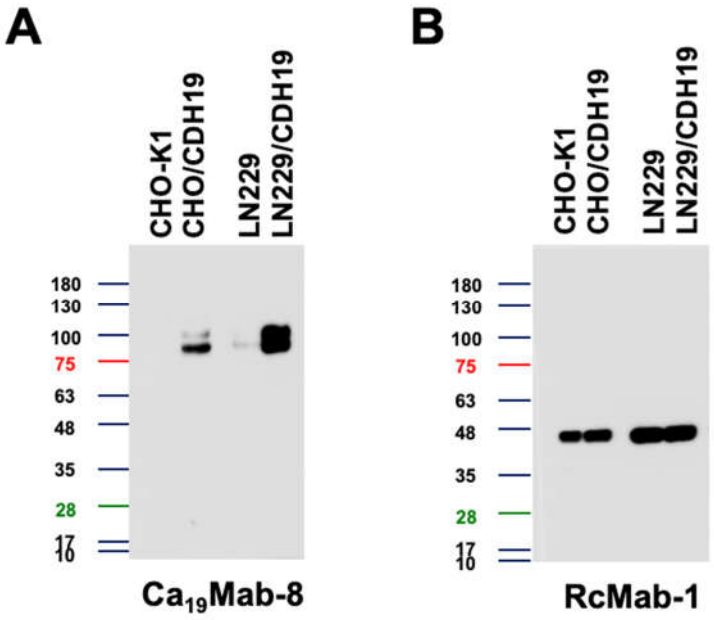

Figure 4. Western blotting using Ca19Mab-8. Cell lysates (10 μg/lane) from CHO-K1, CHO/CDH19, LN229, and LN229/CDH19 were electrophoresed and transferred to polyvinylidene difluoride membranes. The membranes were incubated with 1 μg/mL of Ca19Mab-8 (A) or 1 μg/mL of RcMab-1 (an anti-IDH1 mAb) (B), followed by the treatment with anti-mouse (Ca19Mab-8) or anti-rat IgG (RcMab-1)-conjugated with horseradish peroxidase.

3.5. IHC Using Ca19Mab-8 in Formalin-Fixed Paraffin-Embedded Cell Blocks

We assessed whether Ca19Mab-8 is suitable for immunohistochemistry (IHC) of FFPE sections from CHO-K1 and CHO/CDH19. Ca19Mab-8 showed strong cytoplasmic and membranous staining in CHO/CDH19 but not in CHO-K1 (Figure 5A). Additionally, an anti-PA16 tag mAb, NZ-33, exhibited cytoplasmic and membranous staining in CHO/CDH19, but not in CHO-K1 (Figure 5B). These results suggest that Ca19Mab-8 can detect CDH19 in IHC of FFPE sections of cultured cells.

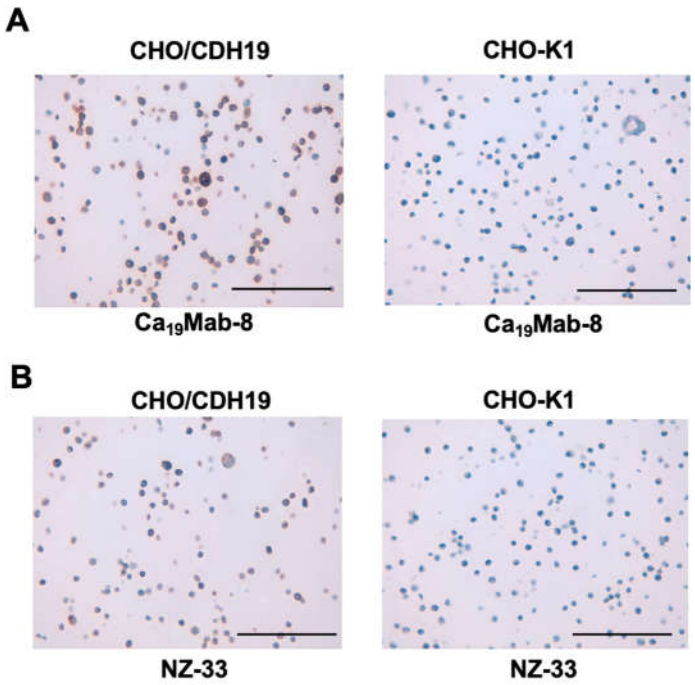

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Figure 5. Immunohistochemistry using Ca19Mab-8 in formalin-fixed paraffin-embedded cell blocks. CHO/CDH19 and CHO-K1 sections were treated with 0.2 μg/mL of Ca19Mab-8 (A) or 0.01 μg/mL of NZ-33 (B). The staining was performed using *BenchMark* ULTRA *PLUS with* the ultraView Universal DAB Detection Kit, Scale bar = 100 μm.

3.5. IHC Using Ca19Mab-8 in Formalin-Fixed Paraffin-Embedded Tumor Tissue

We next performed IHC of Ca19Mab-8 using glioblastoma and melanoma tissue arrays. Ca19Mab-8 showed membranous staining in a case of rectrum malignant melanoma, but isotype control mAb did not (Figure 6 and Table 2). We could not observe positive cases in a glioblastoma tissue array (Supplementary Figure 1). This result indicates that Ca19Mab-8 is suitable to detect endogenous CDH19 in FFPE tumor tissues by IHC.

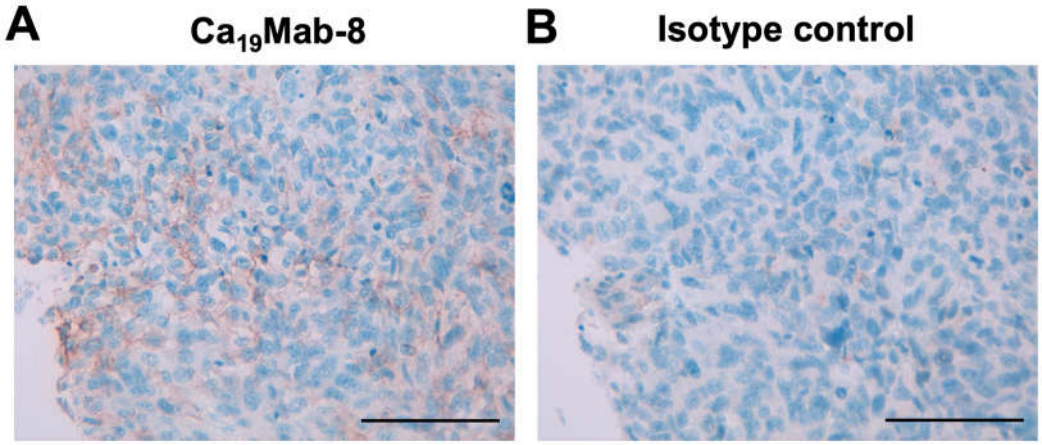

Figure 6. Immunohistochemistry using Ca19Mab-8 in formalin-fixed paraffin-embedded melanoma tissue array. A human melanoma tissue array was treated with 2 μg/mL of Ca19Mab-8 (A) or 2 μg/mL of isotype control IgG1 (CvMab-62) (B). The staining was performed using *BenchMark* ULTRA *PLUS with* the ultraView Universal DAB Detection Kit, Scale bar = 100 μm.

Table 2. Immunohistochemistry using Ca19Mab-8 against melanoma tissue array.

Age	Sex	Pathology diagnosis	TNM	Ca19Mab-8
50	M	Malignant melanoma of esophagus	-	-
62	F	Malignant melanoma of right thumb	T4N0M0	-
70	F	Malignant melanoma of parotid gland	T4N0M0	-
57	M	Malignant melanoma of rectrum	-	+
67	F	Malignant melanoma of rectrum	-	-
70	F	Malignant melanoma of anus	T4N0M0	-
66	M	Malignant melanoma of rectrum	-	-
54	F	Malignant melanoma of rectrum	-	-
82	F	Malignant melanoma of rectrum	-	-
52	F	Malignant melanoma of rectrum	-	-
49	F	Cancer adjacent normal chest skin tissue	-	-
50	F	Cancer adjacent normal skin tissue	-	-

-, No staining; +, Positive staining.

4. Discussion

This study identified novel anti-CDH19 mAbs using the CBIS method (Figure 1). Among them, Ca19Mab-8 recognized both exogenous and endogenous CDH19 in flow cytometry and Western blotting (Figure 3 and Figure 4). Ca19Mab-8 specifically binds to CDH19 but not to other type II, type

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I, 7D, truncated, or atypical CDHs (Figure 2). In contrast, the other nine Ca19Mabs showed cross-reactivity with CDH7 and other type II CDHs in flow cytometry (Table 1). Since most commercially available mAbs lack information on cross-reactivity, careful handling and interpretation are essential when using them. Identifying the Ca19Mab-8 epitope is crucial for developing more specific anti-CDH19 mAbs. Additionally, Ca19Mab-8 is suitable for IHC on cell blocks (Figure 5) and tissue array (Figure 6). IHC was performed using an automated slide-staining system, allowing for standardized staining conditions. Ca19Mab-8 is highly versatile for both basic research and clinical applications.

We demonstrated that Ca19Mab-8 recognized human GBM LN229 cells in flow cytometry and Western blotting (Figure 3 and Figure 4). Although we examined the reactivity of Ca19Mab-8 in other glioblastoma cell lines, LN229 is the only cell line identified by Ca19Mab-8. In contrast, CDH19 was detected by Western blotting in GBM stem-like cells isolated from fresh GBM samples [23]. We should investigate the reactivity of Ca19Mab-8 with those samples and assess its antitumor efficacy. We previously cloned cDNA of mAb and produced recombinant mouse IgG_{2a}-type mAbs to confer antibody-dependent cellular cytotoxicity (ADCC). These mAbs have been evaluated for the antitumor efficacy in human tumor xenograft models [24,25]. We have cloned the cDNA of Ca19Mab-8, and recombinant Ca19Mab-8 will be produced and evaluated for in vitro ADCC activity and antitumor activity in mouse glioblastoma xenograft models.

Metastatic melanoma remains a major clinical challenge [26,27]. Although therapeutic outcomes have significantly improved since the introduction of immune checkpoint inhibitors, about half of patients with metastatic melanoma do not achieve long-term survival benefits [28,29]. A recent single-cell and spatial multi-omics analysis showed that drug-naive human melanoma biopsies contain cancer cells in a mesenchymal-like (MES) state [30]. Melanoma cells in the MES state exhibit resistance to targeted therapy and immunotherapy and are more frequently found in lesions that do not respond to treatment [30].

Ca19Mab-8 showed a membranous staining of CDH19 in a case of rectrum malignant melanoma (Figure 6). Recently CDH19 was identified as a MES signature regulated by the master transcription factor TCF4. Targeting TCF4 genetically enhances immunogenicity and increases the sensitivity of MES cells to immunotherapy and targeted treatments [30]. Therefore, CDH19 is a promising antigen for targeting drug-resistant melanoma cells in the MES state. Moreover, a patent (US 2025/0084160 A1, Mar.13, 2025) reported that CDH19 is expressed in melanoma cell lines, such as CHL-1, which can serve as a preclinical model for melanoma therapy. Since Ca19Mab-8 can detect CDH19-positive cells by flow cytometry (Figure 3) and IHC (Figure 6) without cross-reactivity (Figure 2), Ca19Mab-8 will be a key foundation for developing various modalities, including antibody-drug conjugates, bispecific antibodies, and chimeric antigen receptor T cells.

Author Contributions: G.L.: Investigation; M.K.K.: Conceptualization; H.S.: Investigation, Writing—review and editing; Y.K.: Conceptualization, Funding acquisition, Project administration, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001) for studies involving animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: All related data and methods are presented in this paper. Additional inquiries should be addressed to the corresponding authors.

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

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