Communication

Development of an Anti-Glypican-1 Monoclonal Antibody G1Mab-28 for Flow Cytometry, Western Blotting, and Immunohistochemistry

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Development of an Anti-Glypican-1 Monoclonal Antibody G1Mab-28 for Flow Cytometry, Western Blotting, and Immunohistochemistry

Haruto Yamamoto, Hiroyuki Suzuki, Tomohiro Tanaka, Mika K. Kaneko and Yukinari Kato *

Department of Antibody Drug Development, Tohoku University Graduate School of Medicine,
2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
* Correspondence: yukinari.kato.e6@tohoku.ac.jp; Tel.: +81-22-717-8207

Abstract: Glypican-1 (GPC1) is a heparan sulfate proteoglycan that plays a critical role in regulating various signaling pathways and tumor development. Overexpression of GPC1 promotes tumor cell proliferation and invasiveness, and is associated with poor clinical outcomes. Therefore, anti-GPC1 monoclonal antibodies (mAbs) have been developed in various modalities for tumor therapy. Here, we developed novel anti-GPC1 mAbs using a flow cytometry-based high-throughput screening approach, the Cell-Based Immunization and Screening (CBIS) method. A clone G1Mab-28 (IgG1, κ) reacted with GPC1-overexpressed Chinese hamster ovary-K1 (CHO/GPC1), but not parental CHO-K1, in flow cytometry. Furthermore, G1Mab-28 recognizes endogenous GPC1-expressing human esophageal squamous cell carcinoma KYSE770 cell line. Furthermore, G1Mab-28 specifically recognized only CHO/GPC1, but not other GPC family-overexpressed CHO-K1. The dissociation constant values of G1Mab-28 for CHO/GPC1 and KYSE770 were determined to be 3.3×10^{-8} M and 4.6×10^{-9} M, respectively. Moreover, G1Mab-28 is suitable for western blotting and immunohistochemistry. G1Mab-28, established by the CBIS method, is versatile for basic research and is expected to contribute to the antibody-based tumor therapy.

Keywords: Glypican-1; monoclonal antibody; Cell-Based Immunization and Screening; flow cytometry; immunohistochemistry

1. Introduction

Glypicans are a significant class of extracellular matrix-associated heparan sulfate (HS) proteoglycans [1]. In mammals, the glypican family consists of six members from glypican-1 (GPC1) to glypican-6 (GPC6), all of which share highly conserved amino acid sequences between species [1]. Despite this conservation, individual glypicans exhibit distinct structural and functional characteristics [2]. The glypican family has attracted significant scientific attention due to its involvement in various signaling pathways [3] and tumor development [4].

The first identified member of the glypican family, GPC1, is present in both a membrane-bound form and an enzymatically cleaved soluble form by the metalloprotease ADAM17 [5] or the lipase Notum [6]. Human GPC1 consists of a core protein comprising 558 amino acids, which is anchored to the cell membrane via a glycosylphosphatidylinositol linkage at the serine-530 (S530) residue. Additionally, three HS chains are attached to the core protein at serine residues, S486, S488, and S490 [7]. These HS moieties recruit several signaling molecules, including hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor A (VEGF-A), and transforming growth factor-β (TGF-β), and facilitate their binding to corresponding receptors [3]. Consequently, GPC1 serves essential functions in regulating various signaling pathways involved in tumor cell proliferation, invasiveness, and tumorigenesis [8,9].

GPC1 expression is significantly upregulated in various tumors. Overexpression of GPC1 is associated with reduced overall survival, relapse-free survival, and/or disease-free survival, and

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chemoresistance in esophageal squamous cell carcinoma (SCC) [10]. Furthermore, the GPC1 overexpression has been reported in gliomas, lung SCC, pancreatic, prostate, and breast cancers. In these studies, a strong correlation has been observed between GPC1 overexpression and poor clinical outcomes. [11–15]. Therefore, GPC1 is an important predictive factor for tumor diagnosis.

GPC1 has been evaluated as a potential target for antibody-based therapies, including anti-GPC1 monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), radiotherapy, photoimmunotherapy, chimeric antigen receptor (CAR) T cell therapy, and bispecific T-cell engager (BiTE) in preclinical and clinical studies [7]. To develop these formats, anti-GPC1 mAbs with high reactivity and specificity in flow cytometry are essential.

Using the Cell-Based Immunization and Screening (CBIS) method, various mAbs against membrane proteins, such as chemokine receptors [16–18] and receptor tyrosine kinases [19,20] have been developed. The CBIS method includes immunizing antigen-overexpressed cells and high-throughput screening using flow cytometry. Therefore, mAbs obtained by the CBIS method are suitable for flow cytometry and tend to recognize conformational epitopes. Furthermore, some of these mAbs also apply to western blotting and immunohistochemistry (IHC). This study employed the CBIS method to develop highly versatile anti-GPC1 mAbs.

2. Materials and Methods

2.1. Cell Lines

Chinese hamster ovary (CHO)-K1, mouse myeloma P3X63Ag8.U1 (P3U1), and human glioblastoma LN229 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). KYSE770 was obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). These cells were maintained as described previously [21].

2.2. Plasmid Construction and Establishment of Stable Transfectants

The cDNAs of GPC3v2 (NM_001164618) and GPC6 (NM_005708) were synthesized by Eurofins Genomics KK (Tokyo, Japan). The cDNAs of GPC2 (NM_152742), GPC4 (NM_001448), and GPC5 (NM_004466) were obtained from RIKEN RBC. The cDNAs of GPC2, GPC3v2 (also known as GPC3), GPC4, and GPC5 were cloned into a pCAG-ble vector. A GPC6 cDNA was cloned into a pCAGzeo-ssnPA16 vector [22]. The expression vector of GPC1 (pCMV6_GPC1, NM_002081) was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The plasmids were transfected into CHO-K1 cells and stable transfectants were established by sorting with an anti-PA16 tag mAb (clone NZ-1 for GPC6) [22], an anti-GPC5 mAb (clone 297716; R&D Systems, Inc., Minneapolis, MN, USA), an anti-GPC4 mAb (clone A21050B; BioLegend, San Diego, CA, USA), an anti-GPC3 mAb (clone SP86; Abcam, Cambridge, UK), an anti-GPC2 mAb (clone CT3; Cell Signaling Technology, Inc., Danvers, MA, USA), and an anti-GPC1 mAb (clone 1019718; R&D systems) using a cell sorter (SH800, Sony Corp., Tokyo, Japan). After sorting, cultivation was performed in a medium containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA, USA) or 0.5 mg/mL of G418 (Nacalai Tesque, Inc.). These GPC-overexpressed CHO-K1 (e.g., CHO/GPC1) clones were finally established.

2.3. Hybridoma Production

The female BALB/cAJcl mice were purchased from CLEA Japan (Tokyo, Japan). The mice were intraperitoneally immunized with LN229/GPC1 cells (1×10^8 cells/injection) and Alhydrogel adjuvant 2% (InvivoGen). After three additional immunizations per week (1×10^8 cells/injection), a booster injection (1×10^8 cells/injection) was administered two days before harvesting the spleen cells from immunized mice. The hybridomas were generated as described previously [16].

2.4. Flow Cytometry

Cells were harvested using 1 mM ethylenediaminetetraacetic acid. The cells were washed with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, blocking buffer) and treated

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with mAbs for 30 minutes at 4°C. The cells were then stained with anti-mouse IgG or anti-rat IgG conjugated with Alexa Fluor 488 (Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 minutes at 4°C. The data were collected using an SA3800 Cell Analyzer and analyzed using FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA).

2.5. Determination of Dissociation Constant Values Using Flow Cytometry

CHO/GPC1 and KYSE770 were treated with serially diluted G1Mab-28. Subsequently, the cells were treated with anti-mouse IgG conjugated with Alexa Fluor 488 (200-fold dilution) for 30 minutes at 4°C. The dissociation constant (K_D) values were determined as described previously [19].

2.6. Western Blotting

Using G1Mab-28 (1 μg/mL), 1019718 (1 μg/mL), or an anti-isocitrate dehydrogenase 1 mAb (RcMab-1 [23]) (1 μg/mL), Western blotting was performed as described previously [19].

2.7. Immunohistochemistry (IHC) Using Cell Blocks

Cells were fixed with 4% paraformaldehyde, and the cell blocks were prepared using iPGell (Genostaff Co., Ltd., Tokyo, Japan) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The formalin-fixed paraffin-embedded (FFPE) cell sections were stained with G1Mab-28 (1 μg/mL) or 1019718 (1 μg/mL) using the ultraView Universal DAB Detection Kit *and BenchMark* ULTRA *PLUS (Roche Diagnostics*, Indianapolis, IN, USA*).*

3. Results

3.1. Development of Anti-GPC1 mAbs Using the CBIS Method

To develop anti-GPC1 mAbs, LN229/GPC1 was used as an antigen. Hybridomas were generated by fusing LN229/GPC1-immunized splenocytes with P3U1 cells. After forming colonies, the supernatants were screened for CHO/GPC1-positive and CHO-K1-negative. Subsequently, anti-GPC1 mAb-producing hybridomas were cloned by limiting dilution. A clone G1Mab-28 (IgG1, κ) was finally established (Figure 1).

3.2. Flow Cytometry Using Anti-GPC1 mAbs

We conducted flow cytometry using the G1Mab-28 and a commercially available anti-GPC1 mAb (clone 1019718) against CHO/GPC1 and CHO-K1 cells. The G1Mab-28 and 1019718 recognized CHO/GPC1 dose-dependently from 10 to 0.01 μg/mL (Figure 2A), but did not recognize CHO-K1 even at 10 μg/mL (Figure 2B). We next examined the reactivity of G1Mab-28 against an endogenous GPC1-expressing cell line, KYSE770. G1Mab-28 showed dose-dependent recognition of KYSE770 (Figure 2C). These results indicate that G1Mab-28 recognizes GPC1 in flow cytometry.

3.3. Specificity of G1Mab-28 Against GPC Family Members

We established other GPC2-6-overexpressed CHO-K1, and the specificity of G1Mab-28 to the GPC family members was determined. As shown in Figure 3, G1Mab-28 recognized CHO/GPC1 and did not react with other GPC-overexpressed CHO-K1. These results indicate that G1Mab-28 is a specific mAb against GPC1 among GPC family members.

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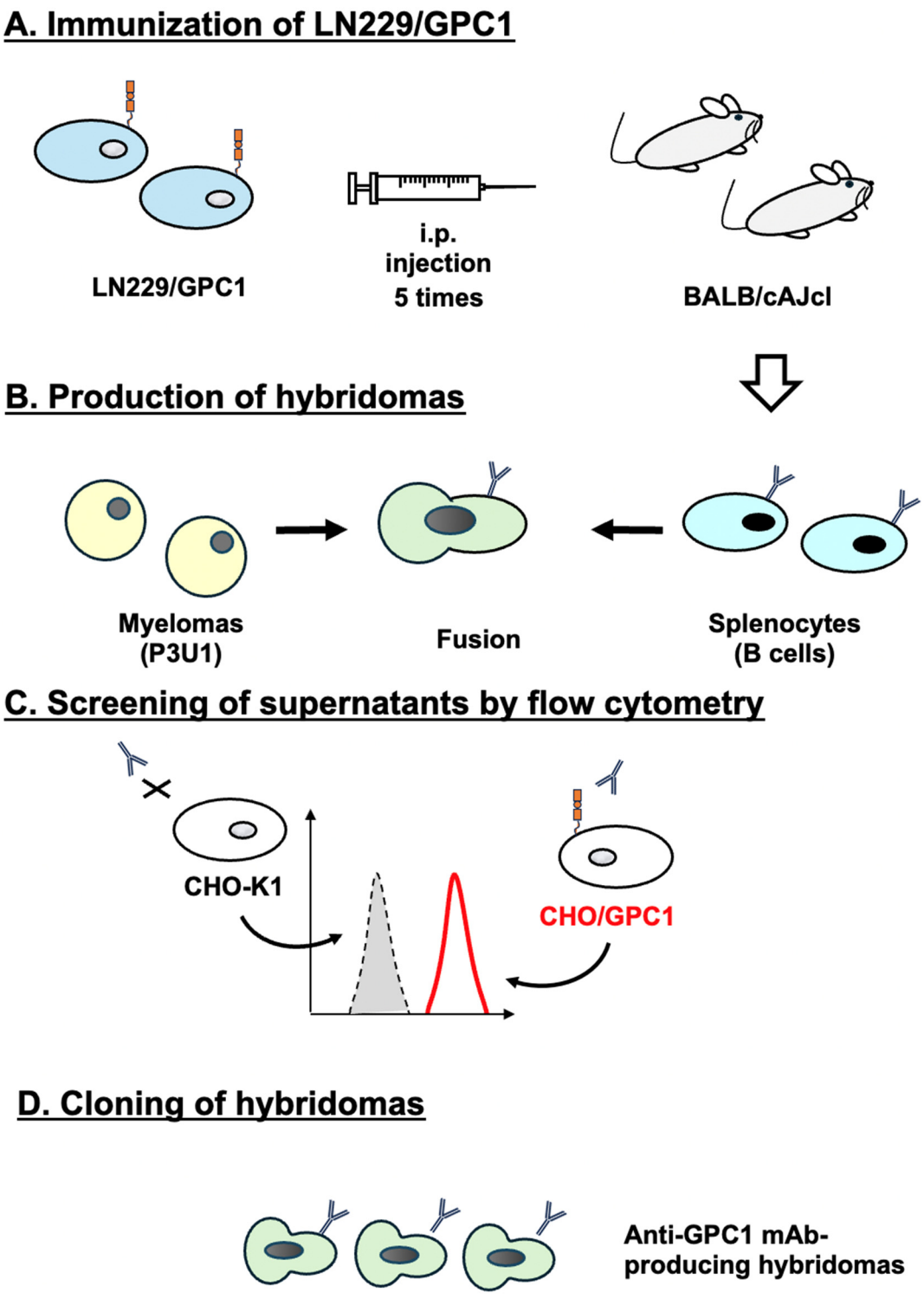

Figure 1. Schematic representation of anti-GPC1 mAbs production. (A) LN229/GPC1 was injected intraperitoneally into BALB/cAJcl mice. (B) After five times immunizations per week, splenocytes were fused with P3U1. (C) A flow cytometry-based high-throughput screening was performed to select the CHO/GPC1-positive and CHO-K1-negative supernatants of hybridomas. (D) Anti-GPC1 specific mAb-producing hybridoma clones were established by limiting dilution.

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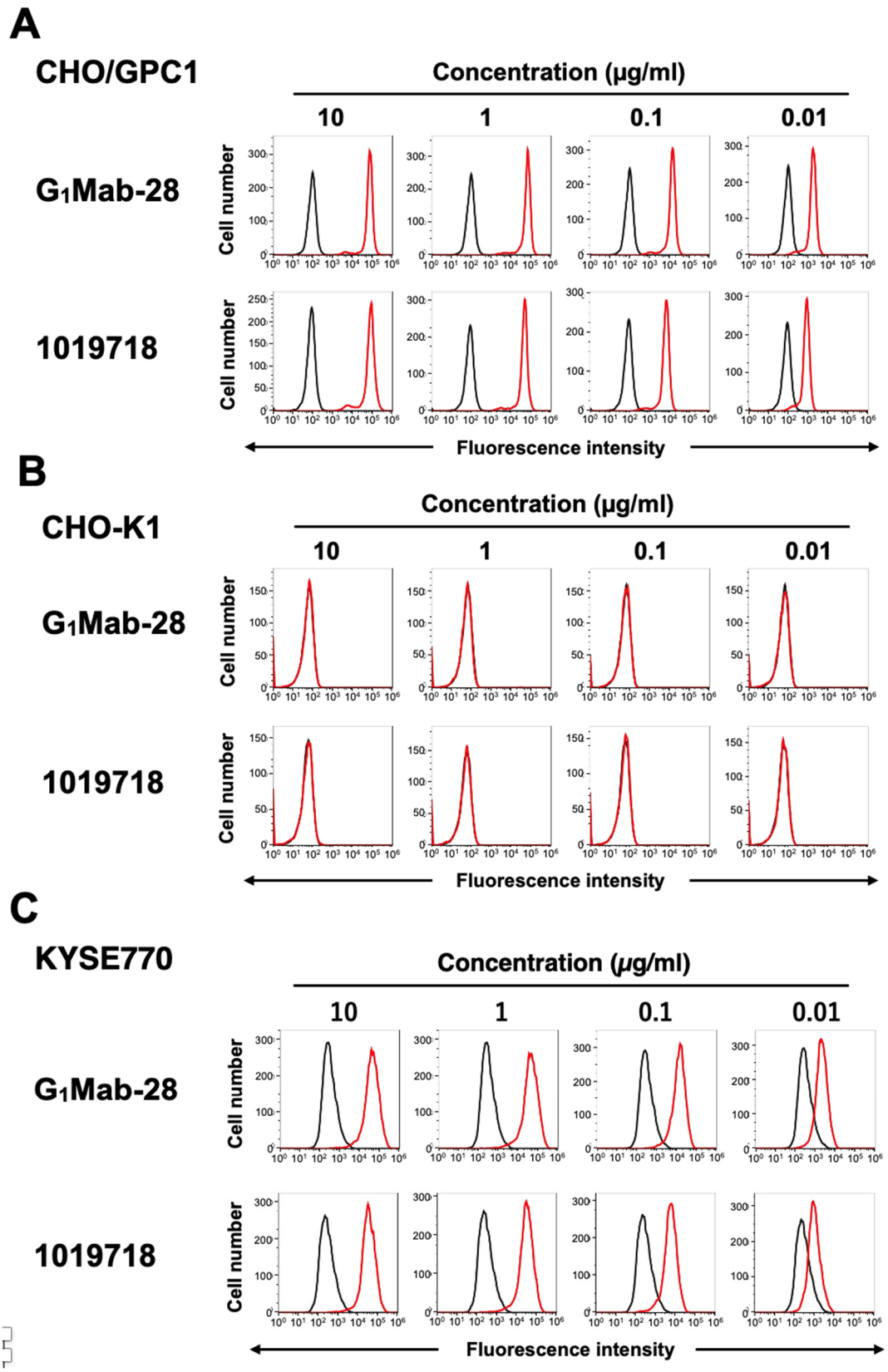

Figure 2. Flow cytometry analysis of G1Mab-28 and 1019718 against CHO/GPC1, CHO-K1, and KYSE770. (A) CHO/GPC1 (A), CHO-K1 (B), and KYSE770 (C) were treated with G1Mab-28 or 1019718 at the indicated concentrations (red) or blocking buffer (black). The mAbs-treated cells were incubated with anti-mouse IgG conjugated with Alexa Fluor 488. The fluorescence data were collected using the SA3800 Cell Analyzer.

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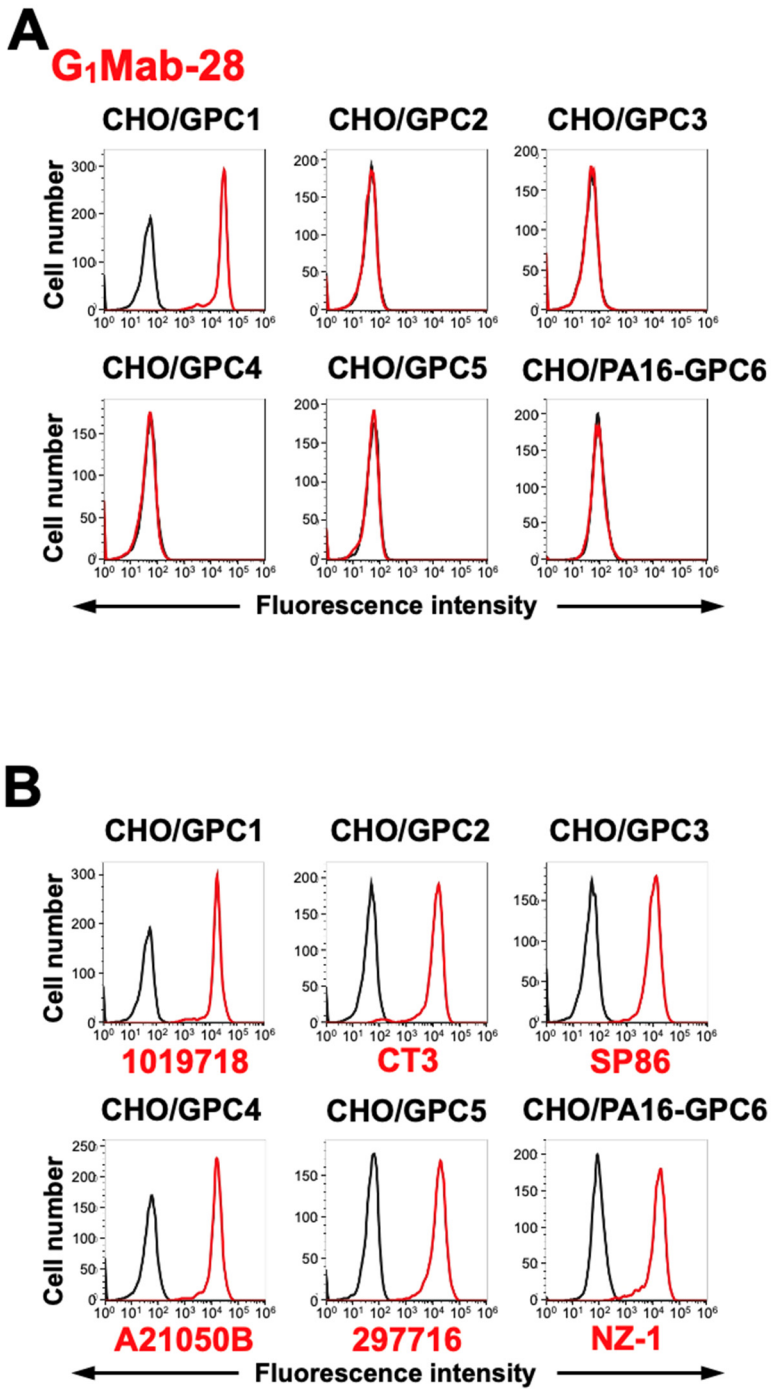

Figure 3. Flow cytometry analysis of G1Mab-28 in GPC family members-expressed CHO-K1 cells. (A) The GPC family members (GPC1, GPC2, GPC3, GPC4, GPC5, and PA16-GPC6)-expressed CHO-K1 cells were treated with 1 μg/mL of G1Mab-28 (red) or control blocking buffer (black), followed by treatment with anti-mouse IgG conjugated with Alexa Fluor 488. (B) The expression of each GPC was confirmed by 1 μg/mL of an anti-GPC1 mAb (clone 1019718), 1 μg/mL of an anti-GPC2 mAb (clone CT3), 1 μg/mL of an anti-GPC3 mAb (clone SP86), 1 μg/mL of an anti-GPC4 mAb (clone A21050B), 1 μg/mL of an anti-GPC5 mAb (clone 297716), and 1 μg/mL of an anti-PA16 mAb, NZ-1.

3.4. Determination of KD Values of G1Mab-28 by Flow Cytometry

The binding affinity of G1Mab-28 was determined using a flow cytometry-based assay. The K_D values of G1Mab-28 for CHO/GPC1 and KYSE770 were 3.3×10^{-8} M and 4.6×10^{-9} M, respectively

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(Figure 4). The K_D values of 1019718 for CHO/GPC1 and KYSE770 were 6.3×10^{-8} M and 1.3×10^{-8} M, respectively (Figure 4). These results indicated that G1Mab-28 has high affinity to GPC1-positive cells.

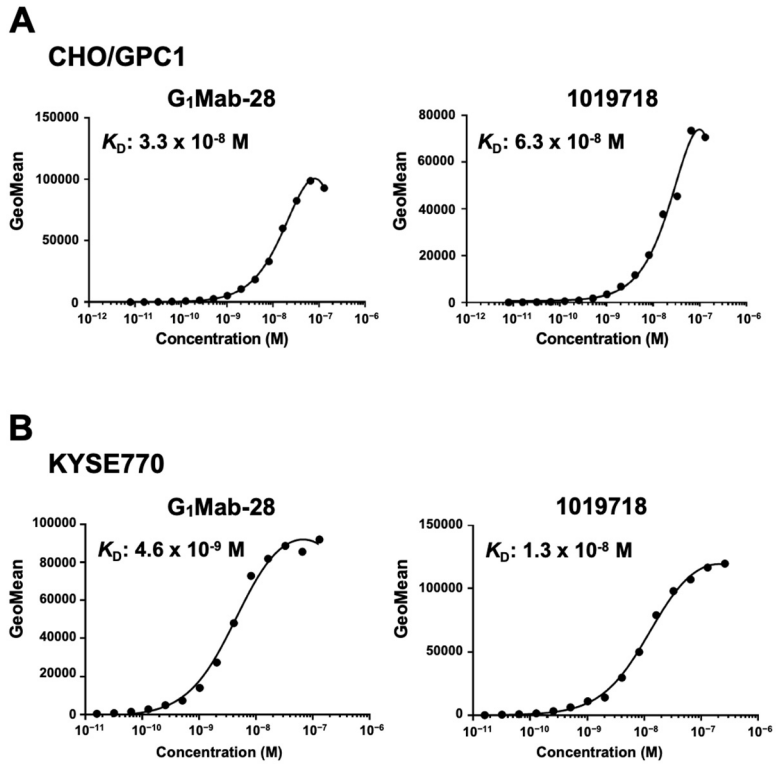

Figure 4. Measurement of binding affinity of G1Mab-28. CHO/GPC1 (A) and KYSE770 (B) were treated with serially diluted G1Mab-28 or 1019718, followed by anti-mouse IgG conjugated with Alexa Fluor 488. The fluorescence data were analyzed using the BD FACSlylic. The K_D values were determined using GraphPad PRISM 6.

3.5. Western Blotting Using G1Mab-28

We next investigated whether G1Mab-28 is suitable for western blotting. Whole-cell lysates of CHO-K1 and CHO/GPC1 were used. G1Mab-28 detected weak bands around 75 kDa in CHO/GPC1, but not in CHO-K1 (Figure 5A, left). 1019718 potently detected in same exposure conditions (Figure 5B). In a long exposure, G1Mab-28 could detect clear bands around 75 kDa in CHO/GPC1, but not in CHO-K1 (Figure 5A, right). IDH1 detected by RcMab-1 was used as an internal control (Figure 5C). These results indicate that G1Mab-28 can detect overexpressed GPC1 in western blotting.

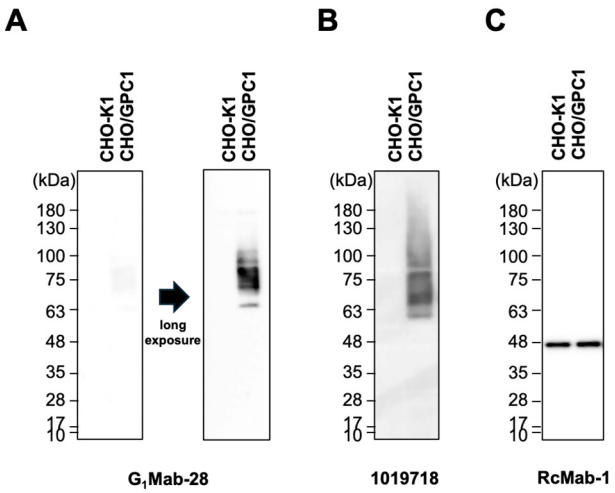

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Figure 5. Western blotting using G1Mab-28. The cell lysate of CHO-K1 and CHO/GPC1 was electrophoresed and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with 1 μg/mL of G1Mab-28 (A), 1 μg/mL of 1019718 (B), or 1 μg/mL of RcMab-1 (an anti-isocitrate dehydrogenase 1, C), followed by treatment with anti-mouse or anti-rat IgG conjugated with horseradish peroxidase. Note that the exposure time of the left side of A and B was the same.

3.6. IHC Using G1Mab-28 in FFPE Cell Sections

We examined whether G1Mab-28 is suitable for the IHC analysis of FFPE sections of CHO-K1 and CHO/GPC1. Both intense membranous and cytoplasmic staining by G1Mab-28 were detected in CHO/GPC1 but not in CHO-K1 (Figure 6A). 1019718 also reacted with CHO/GPC1, but not CHO-K1 (Figure 6B). These results indicate that G1Mab-28 can detect GPC1 in FFPE cell samples.

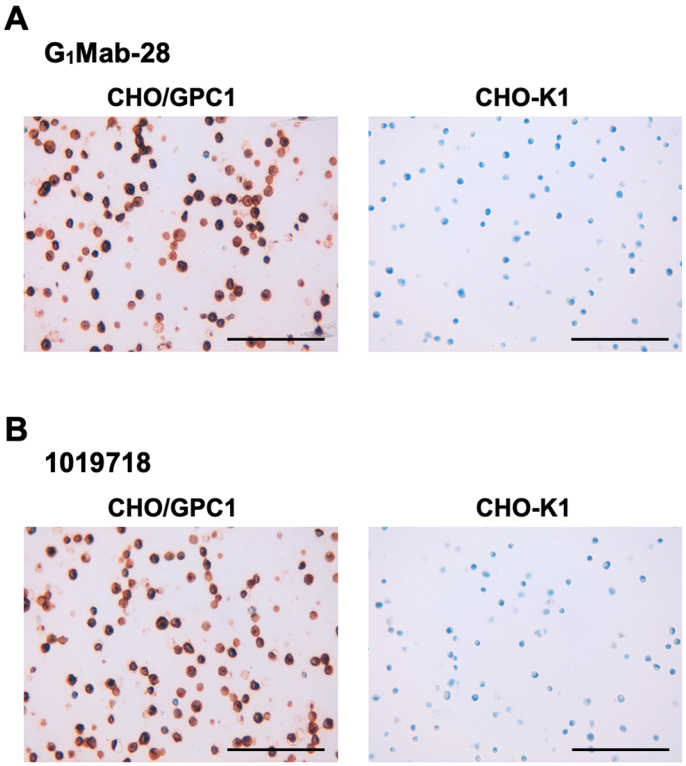

Figure 6. Immunohistochemistry using G1Mab-28 in formalin-fixed paraffin-embedded cell blocks. CHO/GPC1 and CHO-K1 sections were treated with 1 μg/mL of G1Mab-28 (A) or 1 μg/mL of 1019718 (B). The staining was performed using ultraView Universal DAB Detection Kit *and BenchMark* ULTRA *PLUS*. Scale bar = 100 μm.

4. Discussion

This study demonstrated the property of a novel anti-GPC1 mAb clone G1Mab-28 established by the CBIS method. In flow cytometry, G1Mab-28 exhibited the superior reactivity (Figure 2) and affinity (Figure 4) compared to 1019718, a commercially available anti-GPC1 mAb. G1Mab-28 specifically recognized GPC1 among GPC family (Figure 3). Furthermore, G1Mab-28 can detect GPC1 in western blotting (Figure 5) and GPC1-positive cells in immunohistochemistry (Figure 6). Therefore, G1Mab-28 can detect GPC-1 on tumor cells and probably exosomes. It should be determined whether G1Mab-28 can detect an enzymatically cleaved soluble form. The combined detection of exosomal GPC1, exosomal CD82, and serum CA19-9 was reported as a promising method for pancreatic cancer detection [24]. Therefore, G1Mab-28 could contribute to the detection of exosomal GPC1 in early diagnosis of pancreatic cancer.

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GPC1 acts as a co-receptor for HGF, FGF-2, some Wnt ligands, and TGF-β to enhance the signaling pathways, which plays essential roles in tumor cell proliferation, stemness, invasiveness, and epithelial-to-mesenchymal transition [3,9,25]. A GPC1-targeted immunotoxin inhibits pancreatic tumor growth via degradation of internalized GPC1 and downregulation of Wnt signaling [26]. Therefore, the antibody-mediated internalization of GPC1 could reduce the recruitment of those signaling molecules and suppress the tumorigenic signaling. We have developed other clones of anti-GPC1 mAbs (see below). The analyses of their internalizing activity and growth inhibitory effect are essential to evaluate the anti-proliferative effect and future development of ADC.

Several mAbs have been evaluated in preclinical and clinical studies [7]. A chimeric antibody Miltuximab is developed from a BLCA-38 subclone, MIL-38 [7]. The anti-GPC1 mAb, BLCA-38 was obtained by the immunization with human bladder cancer UCRU-BL-17CL cells [27]. A first-in-human trial of Miltuximab was conducted and has validated the safety and tolerability in patients with advanced pancreatic, prostate, and bladder cancer (ACTRN12616000787482) [28]. Furthermore, Miltuximab has been developed to an immunotheranostics ([^{67}Ga]Ga-DOTA-Miltuximab) and evaluated the safety and tolerability in patients with advanced solid cancers [29]. ^{89}Zr-DFO-Miltuximab has been developed as an effective immuno-positron emission tomography imaging agent for detecting GPC1-positive glioblastoma in a mouse model [30]. ^{225}Ac- and ^{177}Lu-labeled Miltuximab have been developed as α- and β-therapy, respectively [7,31]. A photoimmunotherapy, Miltuximab®-IR700 showed significant reduction in viability of GPC1-positive cancer cell lines [32]. MIL-38-CD3 BiTE possesses a tandem scFv format by combining the scFv of Miltuximab® linked to the anti-CD3 scFv. The MIL-38-CD3 BiTE redirects T cell cytolytic activity to kill GPC1-expressing prostate cancer cells in a preclinical model [33]. Other anti-GPC1 mAb-based format such as ADC has been evaluated and exhibited the antitumor efficacy in mouse models of glioblastoma, cholangiocarcinoma, uterine cervical cancer, and pancreatic cancer [34–37]. Anti-GPC1 CAR T cell therapy has been evaluated and exhibited the antitumor efficacy in mouse models of pancreatic cancer [38].

A significant proportion of patients exhibit elevated or detectable GPC1 expression, while the GPC1 expression is thought to be minimal or absent in normal tissues. The expression of GPC1 in normal tissue was evaluated by IHC [4,39]. However, our flow cytometry analysis revealed that both G$_1$Mab-28 and 1019718 recognize non-tumorigenic keratinocyte HaCaT, embryonic fibroblast KMST-6, and embryonic kidney 293FT (Supplementary Figure S1). To attain a favorable therapeutic index while minimizing on-target toxicity, antigens that are highly expressed in tumors but exhibit minimal or no expression in normal tissues are considered ideal targets. Nevertheless, the scarcity of such ideal tumor-associated antigens poses a substantial challenge in the development of therapeutic mAbs for tumor therapy [40].

To address this challenge, we have developed cancer-specific mAbs (CasMabs) targeting various antigens, including human epidermal growth factor receptor 2 (HER2) [41,42], podoplanin [43–45], and podocalyxin [46], and have characterized both their cancer-specific epitopes and recognition mechanisms. In the development of anti-HER2 CasMabs, we generated over 300 anti-HER2 mAb clones by immunizing mice with HER2 expressed on cancer cells. These mAbs were screened for differential reactivity against HER2-expressing tumor versus normal cells using flow cytometry [41]. Among the clones, H$_2$Mab-250 (also referred to as H$_2$CasMab-2) selectively recognized HER2 in breast cancer cells, but not in normal epithelial cells derived from mammary gland, kidney proximal tubule, colon, or lung bronchus [41]. Furthermore, mouse IgG$_{2a}$ type or humanized H$_2$Mab-250 exhibited ADCC, complement-dependent cytotoxicity (CDC), and in vivo antitumor activity against human breast cancer xenografts [47–49]. We have established more clones producing anti-GPC1 mAbs and listed our WEB page "Antibody bank (http://www.med-tohoku-antibody.com/topics/001_paper_antibody_PDIS.htm#GPC1)". We will obtain more than one hundred clones and compare the reactivity to normal and cancer cell lines. The selection of anti-GPC1 CasMabs and identifying the epitopes are essential strategies for developing a novel therapeutic anti-GPC1 CasMab in the future.

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Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Haruto Yamamoto: Investigation. **Hiroyuki Suzuki:** Writing—original draft. **Tomohiro Tanaka:** Investigation, Funding acquisition. **Mika K. Kaneko:** Conceptualization. **Yukinari Kato:** 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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