Establishment and Application of Anti-CD20 Monoclonal Antibodies using the Cell-based Immunization and Screening Method for the Detection of B Cells

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General abstract

In the field of Biochemistry and Medicine, there is immense importance of specific antibodies against a target protein, and thus, a number of such antibodies have been generated, with varied properties. In general, the hybridoma method developed by Kohler and Milstein is used for antibody production. However, it is difficult to produce a few types of antibodies, such as those against proteins with complex structures and multiple transmembrane proteins, using this method. CD20, the target of this study, is one such protein.

CD20 is a small protein with four membrane-spanning domains and two regions consisting of 50 amino acid exposed to the outside of the cell. Since CD20 is specifically expressed on B cells, it is used as a marker for the detection of B cells and is a target molecule for antibody drugs used in B-cell lymphoma. Owing to its importance, several anti-CD20 antibodies have been developed. However, there is still a bottleneck: no single antibody developed till date can be used for many varied applications. For example, antibodies such as rituximab used in lymphoma treatment recognize the extracellular region of CD20, while antibodies used for immunohistochemical analyses,

such as L26, recognize the intracellular region. Due to the difference in the epitopes of these antibodies, there may be a difference in diagnostic results and therapeutic effects when CD20 is mutated. Thus, using several antibodies to analyze a target is an obstacle to obtaining accurate data, because of differences in the epitope and reactivity of the antibodies. However, there are many antibodies, other than anti-CD20 antibodies, that also have limited applications. Therefore, development of antibodies that can be used in various applications, as well as, the methods for producing such antibodies are very important.

In this study, I aimed to develop anti-CD20 antibodies that can be used in different applications, such as flow cytometry (FCM), western blot (WB), and immunohistochemistry (IHC). In addition, this study also used the Cell-Based Immunization and Screening (CBIS) method, a novel method for antibody production, and tested whether it is useful for producing antibodies for difficult targets and multiple applications.

Upon antibody production, 180 of the 7680 samples tested were found to be positive in the first screening, from which 56 clones of monoclonal antibodies were

established. These 56 clones were then used to perform WB and IHC against CD20, of which 13 clones showed reactivity in FCM, WB, and IHC. Two of these clones, C_{20}Mab-11 (Immunoglobulin [Ig]M, kappa) and C_{20}Mab-60 (IgG2a, kappa), were then used for further analysis. Upon doing so, it was found that C_{20}Mab-11 and C_{20}Mab-60 are useful for FCM and WB in endogenous CD20-expressing cell lines, such as BALL-1 (acute lymphoblastoid leukemia cell line) and Raji (Burkitt lymphoma cell line). Upon carrying out IHC of lymph nodes, both the antibodies reacted strongly with B cells in the lymphoid follicles. However, on carrying out analysis of 38 sections of B-cell lymphoma, C_{20}Mab-11 showed reactivity in only 2 of the 38 sections, while C_{20}Mab-60 showed reactivity in 35 sections. These results suggested that C_{20}Mab-11 and C_{20}Mab-60 can be used in FCM, WB, and IHC, and are useful for CD20 research. In addition, C_{20}Mab-60 detected CD20 with high sensitivity when applied for IHC. Epitope analysis revealed that C_{20}Mab-60 recognizes the same region as the anti-CD20 antibody used in the treatment of lymphoma. This suggests that C_{20}Mab-60 may be particularly useful for IHC-based lymphoma analysis. Thus, the antibody production study resulted in the generation of several antibodies against CD20, and many of these antibodies had multi-use properties.

Therefore, the CBIS method can produce antibodies against difficult proteins and is considered to be a useful technique for boosting research in Biochemistry and Medicine.

Abbreviations

mAb: monoclonal antibody

ADCC: antibody-dependent cell-mediated cytotoxicity

B-ALL: B-lymphoblastic leukemia

B-LBL: B-lymphoblastic lymphoma

BSA: bovine serum albumin

CBIS: cell-based immunization and screening

CDC: complement-dependent cytotoxicity

CHO: chinese hamster ovary

DAB: 3,3'-diaminobenzidine tetrahydrochloride

DMEM: Dulbecco’s modified Eagle medium

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

FCM: flow cytometry

FFPE: formalin-fixed paraffin-embedded

Ig: immunoglobulin

IHC: immunohistochemical

NHL: non-Hodgkin's lymphoma

P3U1: P3X63Ag8U.1 cells

PBS: phosphate-buffered saline

RPMI: Roswell Park Memorial Institute

WB: western blot

General introduction

Antibodies play an important role in a variety of fields because of their ability to bind to specific molecules. In Biochemistry research, they are used to identify the expression and localization of specific proteins, and in Medical use, they are used to treat cancer and autoimmune diseases. Thus, many types of antibodies and several methods for producing monoclonal antibodies (mAbs) have been developed.

The first method for producing mAbs was the hybridoma method developed by Kohler and Milstein (1). In this method, B cells from animals immunized with the antigen of interest were fused with myeloma cells to produce hybridomas. This method has made it possible to obtain immortalized cells that stably produce specific antibodies. The desired mAbs can be obtained by screening the antibodies secreted from these cells. Another method is the phage display method developed by Smith and Winter (2, 3). In this method, various antibody genes are introduced into bacteriophages, for expression of antibodies on the surface of the phage, following which the binding of the target antigen to the phage is checked to screen for the target antibody. This method has made it possible to produce antibodies against antigens that are highly toxic to animals and those with low

immunogenicity. In recent years, a method to obtain human antibodies directly from a single human B cell has also been established (4, 5). This method eliminates the necessity for humanization of antibodies, which is a prerequisite when administering animal antibodies to humans, and enables the rapid development of therapeutic antibodies. Although various antibody production methods have been developed, the hybridoma production method is still widely used, because it an easy method to produce antibodies, by means of direct immunization of animals and does not require special machinery. However, the cell fusion method also has a problem: it is time-consuming and labor-intensive to produce good-quality antigens using this method.

In general, synthetic peptides and recombinant proteins are used as immune antigens. While peptides are easy to synthesize, it is difficult to mimic the three-dimensional structure of proteins. Recombinant proteins, on the other hand, can be made to have a three-dimensional structure, but it takes time to purify them. To solve these problems, a novel antibody production method known as the Cell-Based Immunization and Screening (CBIS) method has been developed (Fig. 1).

In the CBIS method, cell lines are exclusively used for immunization and screening, for the purpose of antibody production. For immunization, the target protein-expressing cells are directly administered to animals such as mice, following which the screening is performed using the same cells, by means of flow cytometry (FCM). This technique enables rapid antibody production, because it does not require the preparation of purified antigens. In addition, since immunization and screening are performed using cells, it is possible to produce antibodies against membrane proteins with an accurate three-dimensional structure, which makes this method particularly effective for this application. Previous studies that have attempted antibody production using the CBIS method have generated antibodies against various membrane proteins such as EpCAM (6), CD19 (7), CD44 (8), PD-L1 (9), and podocalyxin (10), in addition to many other useful antibodies.

In these studies, two new advantages of the CBIS method were identified. First, using CBIS method, it is possible and easy to produce single multi-use antibodies that can be used for various applications such as FCM, western blot (WB), and immunohistochemical analyses (IHC). With conventional antibodies, the available

applications are usually limited, which makes it necessary to use different antibodies for each analysis. In such cases, differences in the epitopes and reactivity of the antibodies prevent the acquisition of accurate data. In particular, when mutations occur in the target protein, antibodies that recognize such epitopes lose reactivity, whereas antibodies that recognize other regions do not change reactivity. This highlights the importance of multi-use antibodies and the methods for their production.

To confirm the usefulness of this method, a comparative study between the CBIS method and the conventional method was conducted. Podoplanin (PDPN) from pig (11), horse (12), bear (13), tiger (14), and whale (15) were used as targets for antibody development. In the CBIS method, each cell line stably expressing PDPN was constructed by introducing PDPN gene into CHO cells, while screening was performed by means of FCM using the same cells. In the conventional method, peptides mimicking the 18 amino acids (aa) of each PDPN were synthesized and used for immunization. Screening was performed by means of enzyme-linked immunosorbent assay using the same peptides. Upon antibody production using the conventional method, most of the obtained antibodies could be used only for application in FCM. Only one horse PDPN antibody,

PMab-202 (16), could be used in both FCM and WB. However, PMab-202 did not show reactivity in IHC. In contrast, antibody production using the CBIS method yielded multi-use antibodies against all targets, which could be used for application in FCM, WB, and IHC (11-15). This indicates that even multi-use antibodies, which are difficult to produce using conventional methods, can be efficiently produced using the CBIS method.

Second, CBIS can produce cancer-specific monoclonal antibody (CasMab). CasMabs simultaneously recognize cancer-specific glycans and amino acids of target proteins, such as LpMab-23 (17). Thus, these antibodies are reactive only to cancer cells, even if the target protein is expressed in normal cells. It is believed that the CBIS method can be used to produce new antibodies without side effects for cancer treatment.

These results demonstrate the usefulness of the CBIS method. However, there is still a lack of studies on the production of antibodies against multiple transmembrane proteins using the CBIS method. Multiple transmembrane proteins function as receptors for hormones and neurotransmitters and are known to be involved in various diseases, making them important targets in Biochemistry and Medicine. Therefore, multi-use antibodies are required for the detailed analysis of such proteins. However, it is difficult

to produce purified proteins with an accurate three-dimensional structure. Therefore, the production of antibodies against such proteins is difficult, and warrants the development of an efficient method. To date, the only antibody produced against multiple transmembrane proteins using the CBIS method is CD133 (18). CD133 is also an easy target for antibody production because of its three large extramembrane regions (these regions are 79, 254, and 284 aa in size). Therefore, to prove the usefulness of the CBIS method, it is necessary to produce multi-use antibodies against multiple transmembrane proteins, which are difficult to produce. For the same purpose, this study produced antibodies against CD20, a four-transmembrane protein with a small extracellular region.

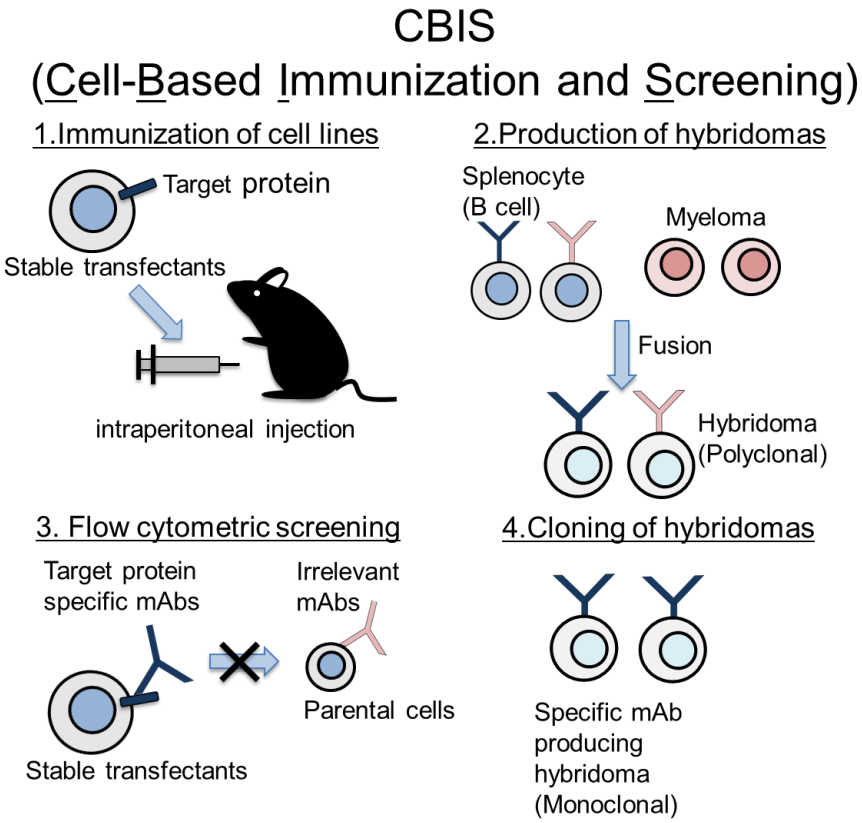


Fig. 1 Schematic illustration of the Cell-Based Immunization and Screening (CBIS) method

Stable transfectants expressing the protein of interest were used as immunogens, with no purification procedure involved. The selection of hybridomas secreting specific mAbs was performed using flow cytometry with parental and transfectant cells.

Chapter 1: Establishment of C_{20}Mab-11, a novel anti-CD20 monoclonal antibody, for the detection of B cells

Abstract

CD20 is one of several B-lymphocyte antigens that is an effective target for the detection and treatment of B-cell lymphomas. Sensitive and specific monoclonal antibodies (mAbs) are required for every application used to diagnose B-cell lymphoma. Although many anti-CD20 mAbs have been established till date, there are limited types of applications that can utilize them. This study aimed to establish novel anti-CD20 mAbs for use in broad applications, such as flow cytometry, western blot, and immunohistochemical analyses, using the Cell-Based Immunization and Screening method. One of the established mAbs, C_{20}Mab-11 (Immunoglobulin [Ig] M, kappa), detected not only the overexpression of CD20 in CHO-K1 or LN229 cell lines, but also endogenous CD20, which is expressed in BALL-1 or Raji lymphoma cell lines. Importantly, C_{20}Mab-11 did not react with CD20-deficient BALL-1 cells, indicating that C_{20}Mab-11 is specific for CD20. In western blot analyses, C_{20}Mab-11 detected CD20 in BALL-1 and Raji cells,

with both high sensitivity and specificity. Furthermore, immunohistochemical analyses carried out using C_{20}Mab-11 resulted in strongly stained B cells of the lymph follicle and B-cell lymphomas. These results indicated that C_{20}Mab-11 is useful for the detection of cells expressing CD20 in lymphoma tissues using flow cytometry, western blot, and immunohistochemical analyses, and could potentially be beneficial for the treatment of B-cell lymphomas.

1. Introduction

CD20 is an integral membrane protein with a molecular weight of 33–37 kDa, which is expressed at high densities only on B lymphocytes (19, 20). CD20 has four membrane-spanning domains and consists of 297 amino acids (aa). The two extracellular domains are located at 72–80 aa and 142–182 aa. The homo-oligomerization of CD20 into tetramers results in a functional calcium channel. CD20 is expressed on B cells, from the stage of pre-B to mature B cell development, and is also detected in many types of non-Hodgkin's lymphoma (21). CD20 is detected in 50% of B-lymphoblastic leukemia/lymphoma originating from pre-B cells, but not in terminally differentiated plasma cell malignancies (22, 23).

The development of sensitive and specific monoclonal antibodies (mAbs) is critical for the diagnosis and treatment of many types of cancer (24). However, the production of sensitive and specific mAbs is a very difficult procedure because it requires the detection of the extracellular loop of multi-pass transmembrane proteins (18). As mentioned above, CD20 possesses two extracellular transmembrane loops that are very small in size. Therefore, compared to the production of mAbs against single-pass

transmembrane proteins, such as CD44 (25) or Programmed death-ligand 1 (9), it is much more difficult to develop sensitive and specific anti-CD20 mAbs for use in multiple applications.

In our previous studies, we developed a Cell-Based Immunization and Screening (CBIS) method, in which cell lines are exclusively used for both immunization and screening. CBIS has previously been used to develop several mAbs against various proteins, including the five transmembrane proteins CD133 (18). Using the CBIS method, we successfully produced sensitive and specific mAbs useful for flow cytometry (FCM), western blot (WB), and immunohistochemical (IHC) analyses (8, 9, 11-15, 18, 27-29).

My objective for developing anti-CD20 mAbs in this study was two-fold. First, CBIS was employed to establish novel anti-CD20 mAbs, with a focus on improving this method for the development of advantageous mAbs against multiple-pass transmembrane proteins. The second objective was the development of multi-use anti-CD20 mAbs that could be used for FCM, WB, and IHC analyses.

2. Materials and Methods

2.1. Cell lines

P3X63Ag8U.1 (P3U1), Chinese hamster ovary (CHO)-K1, Lec1, Lec2, Lec8, and LN229 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Raji and BALL-1 cells were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan). DNA encoding the CD20 gene (IRAL012D02) was provided by RIKEN BRC through the National BioResource Project of MEXT, Japan. The open reading frame of CD20 plus an N-terminal PA-tag (26) was subcloned into a pCAG-Neo or pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). CHO/CD20 was produced by transfecting pCAG-Neo/CD20 into CHO-K1 cells using a Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories, Berkeley, CA, USA). LN229/CD20 was produced by transfecting pCAG-Ble/CD20 into LN229 cells using a Neon™ Transfection System (Thermo Fisher Scientific, Waltham, MA, USA). Lec1/CD20 (*N*-glycan-deficient), Lec2/CD20 (sialic-acid-deficient), and Lec8/CD20 (galactose-deficient) were produced by transfecting pCAG-Ble/CD20 into Lec1, Lec2,

and Lec8 cells, respectively, using the Neon™ Transfection System. The BALL-1/CD20-KO (BINDS-24) cell line was generated by transfecting the cells with CRISPR/Cas9 plasmids for CD20 (Thermo Fisher Scientific) using the Neon™ Transfection System. Stable transfectants were established using the SH800 cell sorter (Sony Corp., Tokyo, Japan).

P3U1, CHO-K1, CHO/CD20, Lec1/CD20, Lec2/CD20, Lec8/CD20, Raji, BALL-1, and BINDS-24 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque Inc., Kyoto, Japan). LN229 and LN229/CD20 cells were cultured in Dulbecco's modified Eagle medium (Nacalai Tesque Inc.). The media were supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific), 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (Nacalai Tesque Inc.). The cells were grown in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.2. Animals

All animal experiments were performed in accordance with relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments described for hybridoma production were approved by the Animal Care and Use Committee of Tohoku University (permit number: 2016MdA-153). The mice were monitored daily for their health. The duration of the experiment was four weeks. Body weight loss exceeding 25% of the total body weight was defined as a humane end-point. Mice were euthanized by means of cervical dislocation, and death was verified in terms of respiratory and cardiac arrest.

2.3. Hybridoma production

Two female BALB/c mice (6-week-old) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under specific pathogen-free conditions. Animal experiments described in the hybridoma production were approved by the Animal Care and Use Committee of Tohoku University (permit number: 2016MdA-153). Briefly, LN229/CD20 cells (1×10^8 cells) were immunized into two BALB/c mice by means of intraperitoneal injection together with Imject Alum (Thermo Fisher Scientific). After

three additional immunizations, a booster injection was administered two days before harvesting the spleen cells. Spleen cells were fused with P3U1 cells using polyethylene glycol 1500 (Roche Diagnostics, Indianapolis, IN, USA), following which the hybridomas were grown in RPMI medium supplemented with sodium hypoxanthine, aminopterin, and thymidine (Thermo Fisher Scientific). The culture supernatants were used for hybridoma screening by means of FCM.

2.4. FCM

Cells were harvested by means of brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque Inc.). After washing with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), cells were treated with 10 μg/mL C_{20}Mab-11 for 30 min at 4°C, followed by treatment with Alexa Fluor™ 488-conjugated anti-mouse Immunoglobulin (Ig) G (1:2000; Cell Signaling Technology, Danvers, MA, USA). Fluorescence data were collected using a Spectral Cell Analyzer SA3800 (Sony Corp.).

2.5. WB analyses

Cell lysates were prepared using 1% Triton™ X-100, following which the cell debris was removed by mans of centrifugation. Cell lysates were boiled in sodium dodecyl sulfate sample buffer with a reducing reagent (Nacalai Tesque Inc.). The proteins (10 μg) were then electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred onto polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque Inc.), the membranes were incubated with 10 μg/mL of C_{20}Mab-11, 1 μg/mL of NZ-1 (anti-PA-tag), or 1 μg/mL of anti-β-actin (clone AC-15; Sigma-Aldrich, St. Louis, MO, USA), followed by incubation with peroxidase-conjugated anti-mouse IgG secondary antibody (diluted 1:1000; Agilent Technologies, Santa Clara, CA, USA) or anti-rat IgG (diluted 1:10,000; Sigma-Aldrich), respectively. Finally, the proteins were detected with ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) using Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

2.6. IHC analyses

One formalin-fixed paraffin-embedded (FFPE) tissue sample from an oropharyngeal squamous cell carcinoma patient who underwent surgery at Sendai Medical Center was used for this study (27). Written informed consent was obtained from the patients for sample procurement and subsequent data analyses. Tissue microarrays (CC00-10-001), including those for lymphomas, normal lymph nodes, and normal thyroid, were purchased from Cybrdi Inc. (Rockville, MD, USA).

The 4-μm thick FFPE tissue sections were directly autoclaved in EnVision™ FLEX Target Retrieval Solution High pH (Agilent Technologies) for 20 min. After blocking with SuperBlock™ T20 (PBS) Blocking Buffer (Thermo Fisher Scientific), the tissue sections were incubated with C_{20}Mab-11 (5 μg/mL) for 1 h at room temperature and treated with the EnVision+ Kit for mouse (Agilent Technologies) for 30 min. The color was developed using 3,3'-diaminobenzidine tetrahydrochloride (Agilent Technologies) for 2 min, while counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

2.7. Enzyme-linked immunosorbent assay (ELISA)

The CD20 peptides synthesized using PEPScreen (Sigma-Aldrich Corp.) were immobilized on Nunc MaxiSorp™ 96-well immunoplates (Thermo Fisher Scientific) at a concentration of 1 mg/mL for 30 min at 37°C. After blocking with 0.1% BSA in PBS, the plates were incubated with 10 μg/mL purified C_{20}Mab-11, followed by incubation with 1:2000 dilution of peroxidase-conjugated anti-mouse IgG (Agilent Technologies). The enzymatic reaction was performed using an ELISA POD Substrate TMB Kit (Nacalai Tesque Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Berkeley, CA, USA). These reactions were performed at 37°C using a total sample volume of 50–100 mL.

2.8. Statistical analyses

Statistical analysis was conducted using *t*-test with Prism 6 (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $P<0.05$. All data have been expressed as mean ± SEM.

3. Results

3.1. Establishment of anti-CD20 mAbs

The CBIS method was employed in this study (Fig. 2). First, two mice were immunized with LN229/CD20 cells, following which their spleen cells were harvested and grown as hybridomas in cell culture. Next, supernatants from the cultured hybridomas positive for CHO/CD20 and negative for CHO-K1 were selected using FCM. Strong signals were detected from CHO/CD20, while weak or no signals were detected from CHO-K1 in 14 of the 960 wells (1.5%). Further screening using WB and IHC techniques led to the establishment of C_{20}Mab-11 (IgM, kappa).

3.2. FCM analyses

Using FCM analysis, I found that C_{20}Mab-11 reacted with CHO/CD20 cells, but not with parental CHO-K1 cells (Fig. 3A). Similarly, C_{20}Mab-11 reacted with LN229/CD20 cells but not with parental LN229 cells. Additionally, FCM was performed using CD20-stable transfectants of glycan-deficient CHO cell lines (Lec1, Lec2, and Lec8), although it was previously reported that CD20 is not glycosylated (30). C_{20}Mab-11 reacted with

Lec1/CD20 (*N*-glycan-deficient), Lec2/CD20 (sialic-acid-deficient), and Lec8/CD20 (galactose-deficient) cells, but not with parental Lec1, Lec2, and Lec8 cells, indicating that the binding epitope of C_{20}Mab-11 is independent of glycans. Furthermore, C_{20}Mab-11 recognized the endogenous CD20 of BALL-1 cells, but did not react with CD20-knockout BALL-1 (BINDS-24). These results indicated that C_{20}Mab-11 is specific for CD20. The geomean of each signal was then quantitatively analyzed (Fig. 3B).

3.3. WB analyses

Next, I performed WB using the C_{20}Mab-11 antibody. C_{20}Mab-11 detected CD20 with a 45-kDa band in CHO/CD20 cells, and a 37-kDa band in BALL-1 and Raji cells; however, it did not detect CD20 in parental CHO-K1 cells or CD20-knockout BALL-1 (BIND-24) cells (Fig. 4), indicating that it is specific for CD20. An anti-PA-tagged mAb detected CD20 at a 45-kDa band, without detecting any bands in CHO-K1, BALL-1, BINDS-24, and Raji cells. The difference in molecular sizes detected between the CHO/CD20 and BALL-1 cells might be due to the N-terminal PA-tagging of CD20. Although additional bands of 30 kDa and 20 kDa were detected by C_{20}Mab-11 and an anti-PA-tag mAb (NZ-

1), respectively, in CHO/CD20 cells, these could be degraded fragments of the CD20 protein.

3.4. IHC analyses

The immunohistochemical utility of C_{20}Mab-11 was further investigated in the lymph node of an oropharyngeal squamous cell carcinoma patient. C_{20}Mab-11 strongly stained the lymph follicles, while it weakly stained the cortex (Fig. 5A-B). No staining was observed without primary antibody (Fig. 5C-D). HE staining was also performed on consecutive SCC tissues (Fig. 5E-F).

C_{20}Mab-11 weakly stained the B cells of normal lymph nodes (Fig. 5G-H) and B cell lymphomas (Fig. 6A-B). No staining was observed in B-cell lymphomas without the primary antibody (Fig. 6C-D). C_{20}Mab-11 (Fig. 6E-F) and control (Fig. 6G-H) did not stain the normal thyroid tissues. These results indicated that C_{20}Mab-11 is useful for detecting B cells in IHC analyses of FFPE tissues.

3.5. Determination of C_{20}Mab-11 epitope using ELISA

To investigate the epitope of C_{20}Mab-11, the extracellular regions of CD20, such as 71-81 aa, 140-159 aa, 150-169 aa, 160-179 aa, 170-189 aa, and 180-189 aa, were synthesized (Table 1) and tested for their reactivity by means of ELISA. While 71-81 aa, 140-159 aa, 150-169 aa, 170-189 aa, 180-189 aa showed no reactivity, 160-179 aa showed reactivity (Fig. 7). In the next step of the study, 20 peptides were prepared, in which each amino acid in the 160-179aa region was replaced with A or G (Table 2), followed by testing of the reactivity of the peptides using ELISA. C_{20}Mab-11 showed similar reactivity to P160A, Y161A, I162A, I164A, Y165A, S167A, N176A, S177A, P178A, and S179A, as it did to the wild-type sequence. In contrast, C_{20}Mab-11 did not react with E168A, P169A, A170G, N171A, P172A, S173A, E174A, and K175A; reacted only moderately with N163A; and reacted weakly with N166A (Fig. 8A). This indicates that the Asn163, Asn166, Glu168, Pro169, Ala170, Asn171, Pro172, Ser173, Glu174, and Lys175 in the CD20 sequence are critical epitopes of C_{20}Mab-11. The results described above are summarized in Fig. 8B.

4. Discussion

In this study, I successfully developed a sensitive and specific anti-CD20 mAb, C_{20}Mab-11, using the CBIS method previously developed in our laboratory (Fig. 2) (18). Because the CBIS method does not require purified proteins for immunization and screening, it is an easier and more effective method to develop mAbs against multi-pass transmembrane proteins. Previously, we developed anti-CD133 mAbs using the CBIS method, without purifying CD133 proteins (18). The anti-CD133 mAbs were determined to be useful for every application, including FCM, WB, and IHC analyses. Although there are several commercially available anti-CD133 mAbs, their applications are usually limited (31), thus requiring the use of several mAbs or polyclonal antibodies for different experimental procedures.

There are many commercially available anti-CD20 mAbs, as shown in Table 3. As listed, most of these anti-CD20 mAbs are usually applicable to only one or two experimental techniques. Although a few mAbs are useful for at least three applications, including FCM, they do not react with the extracellular domains of CD20 because only the intracellular domains were used as the immunogen during the development of these

mAbs. Rituximab [a mouse-human chimeric mAb; the original mouse clone 2B8 (33)] is the first anti-CD20 mAb approved by the US FDA, and is used for the treatment of B-cell NHL or B-cell chronic lymphocytic leukemia (33). Rituximab shows high sensitivity in FCM and exerts high antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and anti-tumor activities. However, rituximab could not detect the CD20 protein in the WB analysis in my study (data not shown). In contrast, C_{20}Mab-11 could detect endogenous CD20 protein, which is expressed in BALL-1 and Raji cells (Fig. 4). Furthermore, C_{20}Mab-11 could stain normal B-cells or B-cell lymphoma cells in IHC analyses using FFPE tissues (Fig. 5 and 6). Because mAbs possess different epitopes, it might be preferable to use the same mAb of the accordant epitope for both diagnosis and therapy. In this study, one oropharyngeal squamous cell carcinoma tissue, one B-cell lymphoma tissue, and two normal human tissues were used for C_{20}Mab-11 immunostaining. In future studies, it would be useful to further validate my results in more tissues with different degrees of disease progression.

C_{20}Mab-11 may also be advantageous for targeting CD20-expressing B-cell lymphomas. My future studies will focus on a different subclass of C_{20}Mab-11 (mouse

IgM), mouse IgG_{2a} or human IgG_1, and determine whether these mAbs also demonstrate ADCC/CDC and anti-tumor activities (10).

Anti-CD20 mAbs in combination with rituximab, ^{131}I-Tositumomab (radio-immunotherapy) (34), ibritumomab tiuxetan (radio-immunotherapy) (35), ofatumumab (a fully human mAb) (36), and obinutuzumab (a humanized glycoengineered mAb) (37), have shown significant survival benefits in B-cell lymphoma patients. In follicular lymphoma, the four-year overall survival rate significantly improved upon using a combination of cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP) therapy and an anti-CD20 mAb (91%), as compared to using CHOP alone (69%) (38). In elderly patients (60–80 years old) with diffuse large B-cell lymphoma, overall survival improved by 13% following treatment with rituximab plus CHOP, as compared to that with CHOP alone (38). Although anti-CD20 mAbs are very effective for the treatment of B-cell lymphoma, many patients do not present a clinical response to anti-CD20 mAbs (39). Furthermore, 60% of NHL relapse patients who initially respond to rituximab show no subsequent clinical response to rituximab, due to the loss of CD20 expression (39). To overcome resistance to anti-CD20 mAbs, there is a need for development of more

effective first-line lymphoma treatments. Future studies will investigate whether C_{20}Mab-11 may be used as an antibody-containing drug to treat CD20-expressing lymphomas.

5. Figures and tables

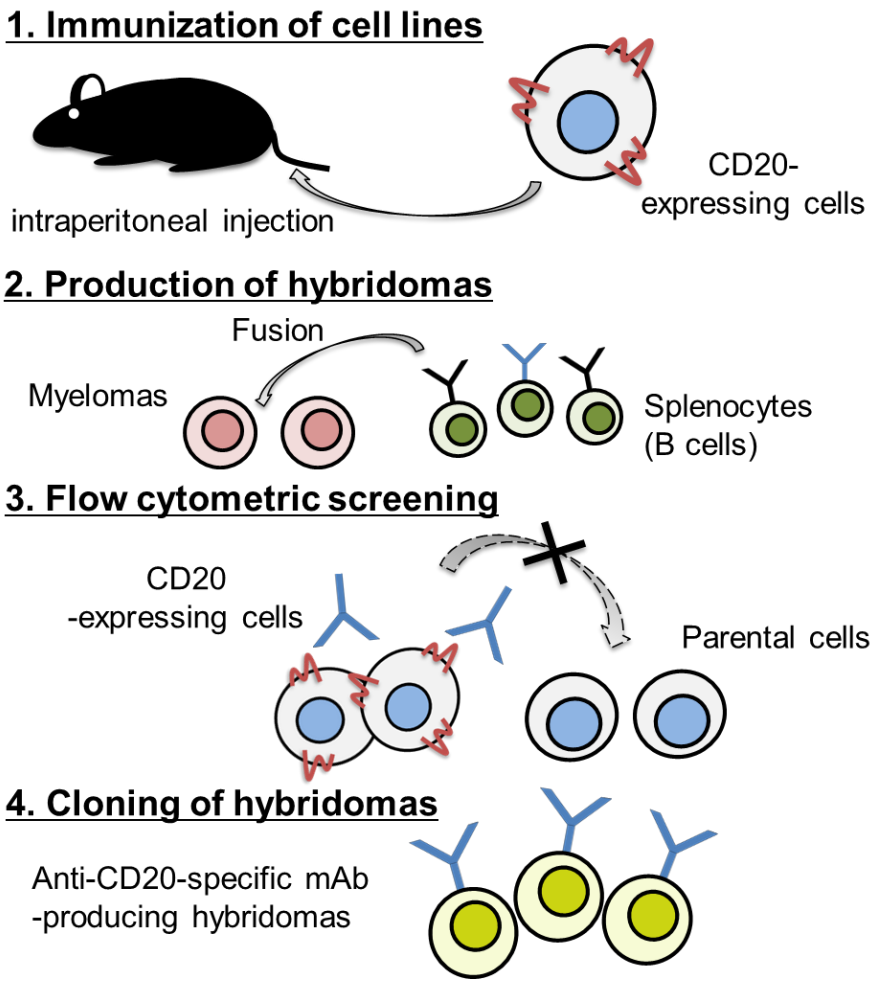


Fig. 2 Production of anti-CD20 mAbs

(A) Procedure for the Cell-Based Immunization and Screening (CBIS) method. LN229/CD20 cells were immunized into BALB/c mice by means of intraperitoneal injection, followed by screening using flow cytometry.

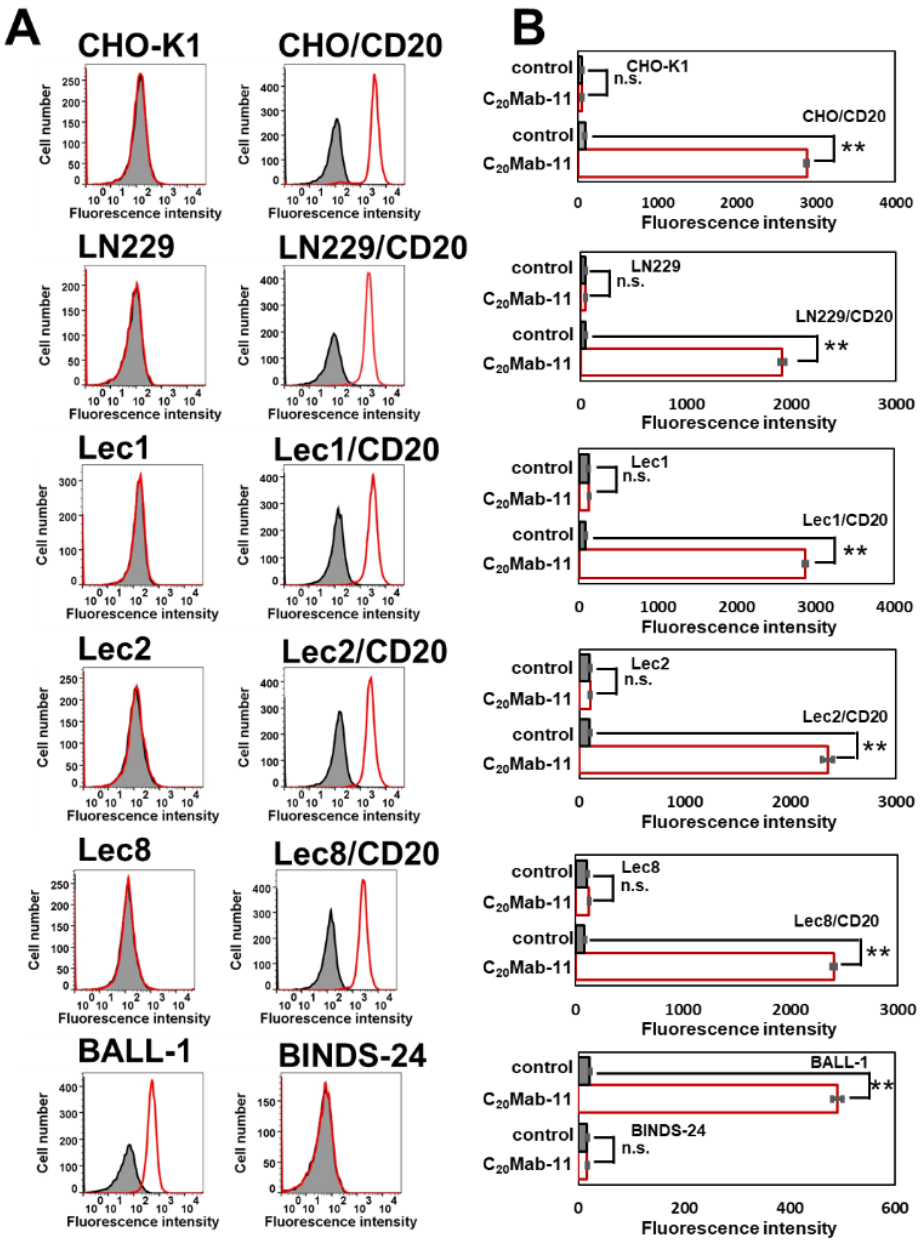


Fig. 3 Flow cytometry analyses using C_{20}Mab-11

(A) CHO-K1, CHO/CD20, LN229, LN229/CD20, Lec1 (*N*-glycan-deficient), Lec1/CD20, Lec2 (sialic acid-deficient), Lec2/CD20, and Lec8 (galactose-deficient), Lec8/CD20, BALL-1, and BINDS-24 (CD20-deficient BALL-1) cells were treated with

C_{20}Mab-11 (red line) at a concentration of 10 μg/mL or 0.1% BSA in PBS (gray) for 30 min, followed by incubation with secondary antibodies. (B) The geomean of each signal was quantified. **P<0.01. All data have been expressed as mean ± SEM.

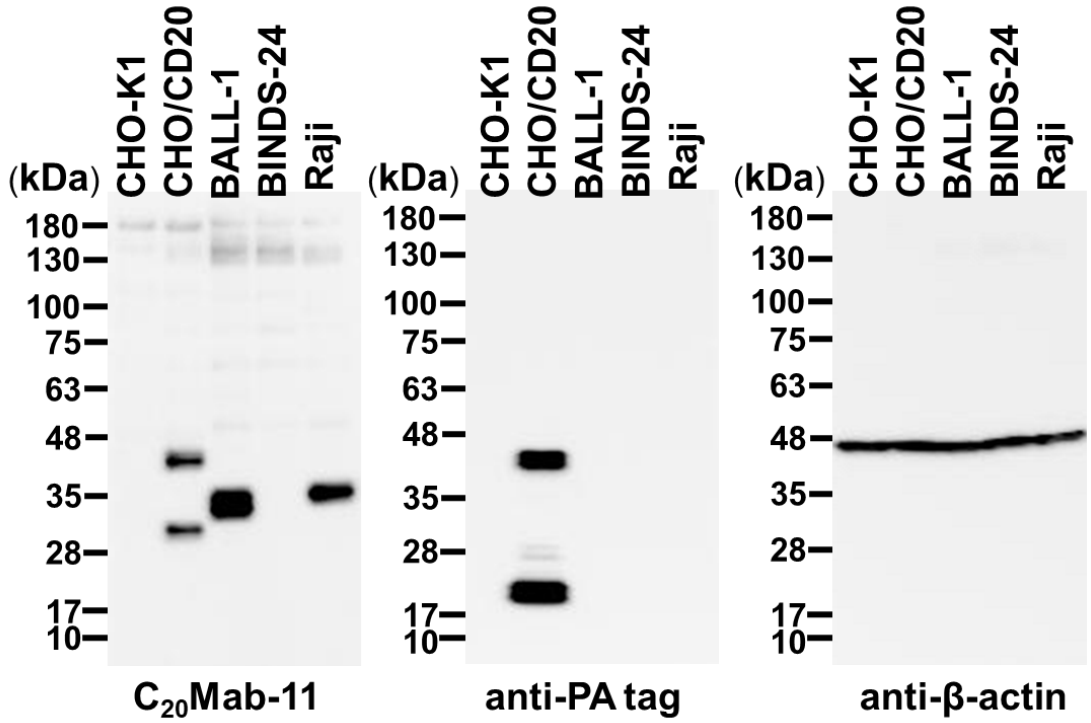


Fig. 4 Western blot using C_{20}Mab-11

Cell lysates of CHO-K1, CHO/CD20, BALL-1, BINDS-24, and Raji cells were electrophoresed and transferred onto PVDF membranes. These membranes were then treated with C_{20}Mab-11 (left panel), NZ-1 (anti-PA-tag; middle panel), or anti-β-actin (right panel), followed by incubation with secondary antibodies.

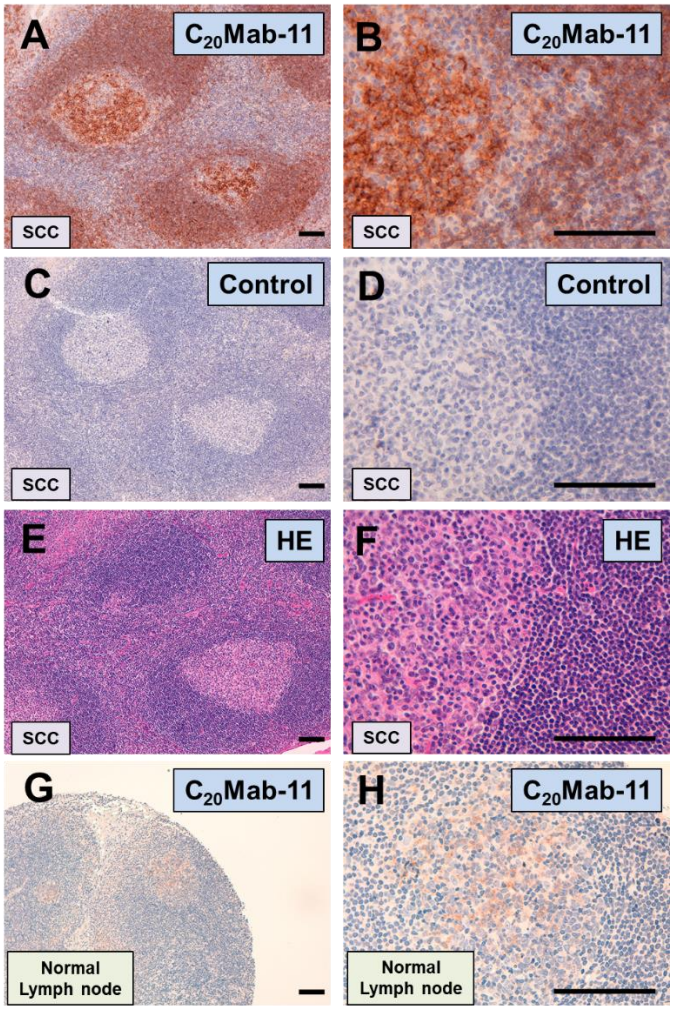


Fig. 5 Immunohistochemical analyses of oropharyngeal squamous cell carcinoma or normal lymph node using C_{20}Mab-11

Consecutive tissue sections of oropharyngeal squamous cell carcinoma (SCC; **A-F**) and normal lymph node tissue sections (**G-H**) were incubated with C_{20}Mab-11 (**A-B and G-H**) or blocking buffer (**C-D**), followed by treatment with EnVision+ kit. Counterstaining was performed using hematoxylin (**A-D and G-H**). Hematoxylin and eosin (HE) staining was also performed on consecutive SCC tissues (**E-F**). Scale bar: 100 μm.

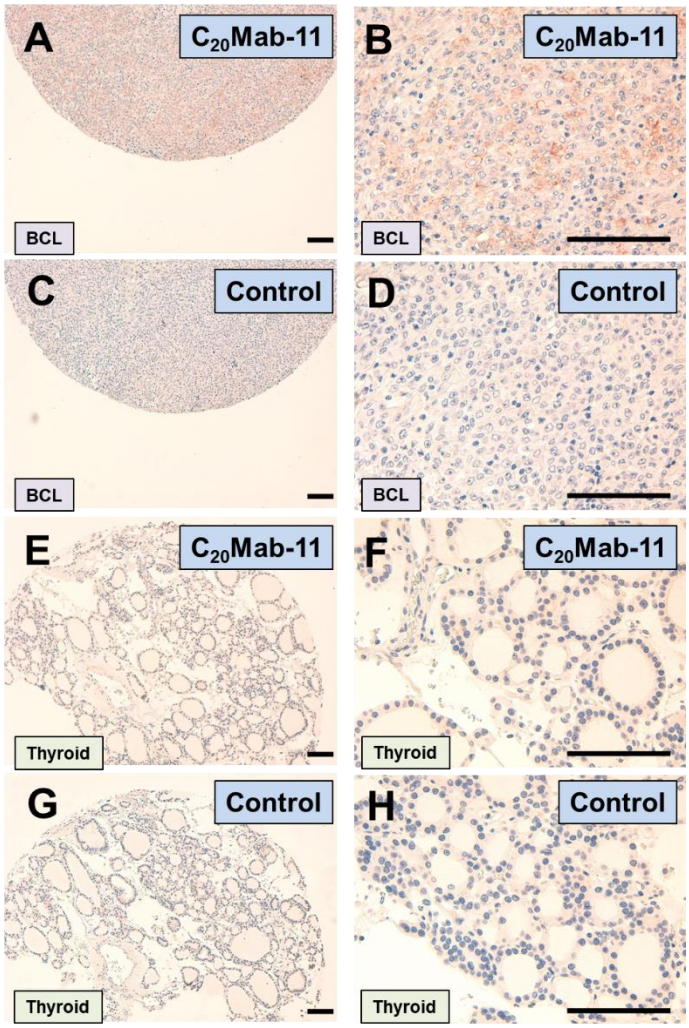


Fig. 6 Immunohistochemical analyses of B-cell lymphomas and B-cells using C_{20}Mab-11

Consecutive tissue sections of B-cell lymphomas (BCL; **A-D**) and normal thyroid tissue sections (**E-H**) were incubated with C_{20}Mab-11 (**A-B and E-F**) or blocking buffer (**C-D and G-H**), followed by treatment with EnVision+ Kit. Counterstaining was performed using hematoxylin (**A-H**). Scale bar: 100 μm.

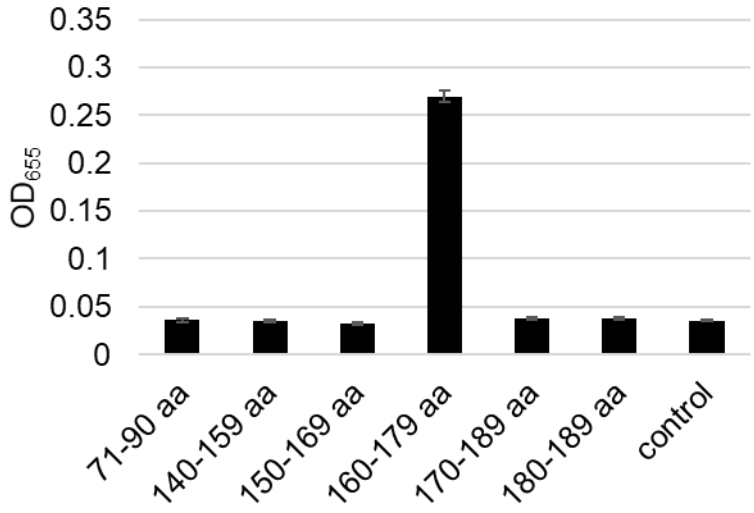


Fig. 7 Determination of the C_{20}Mab-11 epitope for CD20 using ELISA with deletion mutants

The synthesized CD20 peptides were immobilized on immunoplates. The plates were then incubated with C_{20}Mab-11 (10 μg/mL), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins.

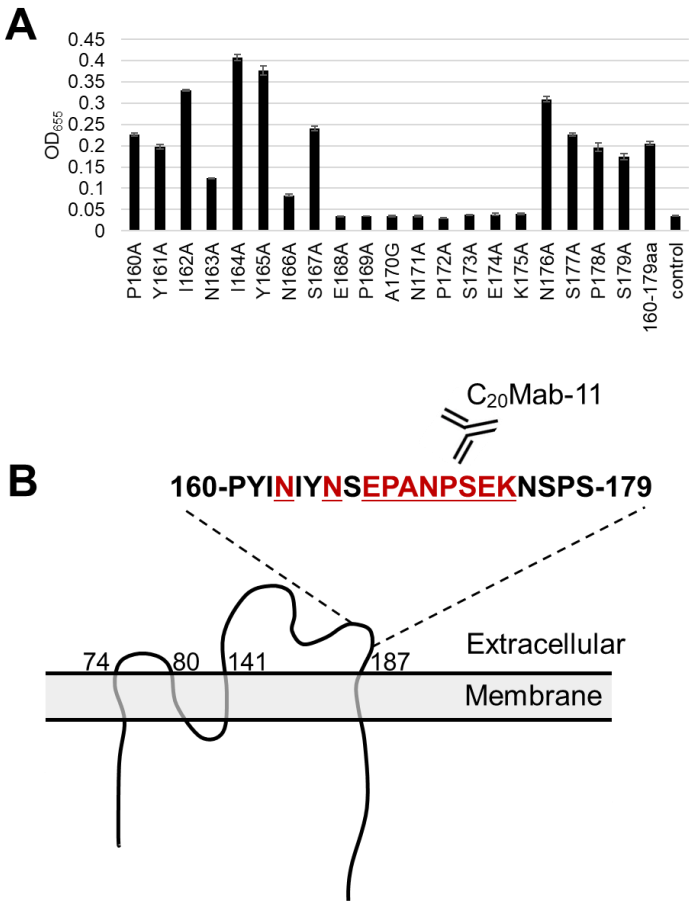


Fig. 8 Determination of the C_{20}Mab-11 epitope for CD20 using ELISA with point mutants

(**A**) Synthesized peptides of CD20 were immobilized on immunoplates. The plates were incubated with C_{20}Mab-11 (10 μg/mL), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins. (**B**) Schematic illustration of CD20 and the C_{20}Mab-11 epitope. The C_{20}Mab-11 epitope of CD20 involves Asn163, Asn166, Glu168, Pro169, Ala170, Asn171, Pro172, Ser173, Glu174, and Lys175.

Table 1. Identification of C_{20}Mab-11 epitope
using synthesized peptides (deletion mutant) by ELISA.

Peptides	*Sequence*	*C_{20}Mab-11*
71-90 aa	MIPAGIYAPISVTVWYPLWG	-
140-159 aa	NIKISHFLKMESLNFIRAHT	-
150-169 aa	ESLNFIRAHTPYINIYNSEP	-
160-179 aa	PYINIYNSEPANPSEKNSPS	+++
170-189 aa	ANPSEKNSPSTQYSYSIQSL	-
180-189 aa	TQYSYSIQSL	-

+++, $OD_{655} \geqq 0.2$; ++, $0.1 \leqq OD_{655} < 0.2$; +, $0.06 \leqq OD_{655} < 0.1$; -, $OD_{655} < 0.06$.

Table 2. Identification of C_{20}Mab-11 epitope using synthesized peptides by ELISA.

Peptides	*Sequence*	*C_{20}Mab-11*
P160A	**A**YINIYNSEPANPSEKNSPS	+++
Y161A	P**A**INIYNSEPANPSEKNSPS	+++
I162A	PY**A**NIYNSEPANPSEKNSPS	+++
N163A	PYI**A**IYNSEPANPSEKNSPS	++
I164A	PYIN**A**YNSEPANPSEKNSPS	+++
Y165A	PYINI**A**NSEPANPSEKNSPS	+++
N166A	PYINIY**A**SEPANPSEKNSPS	+
S167A	PYINIYN**A**EPANPSEKNSPS	+++
E168A	PYINIYNS**A**PANPSEKNSPS	-
P169A	PYINIYNSE**A**ANPSEKNSPS	-
A170G	PYINIYNSEP**G**NPSEKNSPS	-
N171A	PYINIYNSEPA**A**PSEKNSPS	-
P172A	PYINIYNSEPAN**A**SEKNSPS	-
S173A	PYINIYNSEPANP**A**EKNSPS	-
E174A	PYINIYNSEPANPS**A**KNSPS	-
K175A	PYINIYNSEPANPSE**A**NSPS	-
N176A	PYINIYNSEPANPSEK**A**SPS	+++
S177A	PYINIYNSEPANPSEKN**A**PS	+++
P178A	PYINIYNSEPANPSEKNS**A**S	+++
S179A	PYINIYNSEPANPSEKNSP**A**	+++
160-179aa	PYINIYNSEPANPSEKNSPS	+++

Bold ‘‘A’’ or ‘‘G’’ indicates the substitution from original amino acid. +++, $OD_{655} \geqq 0.15$; ++, $0.1 \leqq OD_{655} < 0.15$; +, $0.06 \leqq OD_{655} < 0.1$; -, $OD_{655} < 0.06$.

Table 3. Commercially available anti-CD20 mAbs.

Clone	Company	Speicies	Isotype	Immunogen	Application		
					FCM	IHC	WB
4A7G3	proteintech	mouse	IgG2b	Protein	+ (i.c.)	+	+
EP459Y	abcam	rabbit	IgG	Peptide (i.c.)	+ (i.c.)	+	+
SP32	abcam	rabbit	IgG	Peptide (i.c.)	+ (i.c.)	+	+
L26	abcam	mouse	IgG2a	B cells	+ (i.c.)	+	+
MS4A1/3409	abcam	mouse	IgG2b	Peptide (i.c.)	+ (i.c.)	+	+
MEM-269	genetex	mouse	IgM	RAMOS cells	+	+	-
SPM618	genetex	mouse	IgG2a	Protein	+	+	-
IGEL/1497R	genetex	rabbit	IgG	Protein	+	+	-
E7B7T	CST	rabbit	IgG	Protein	-	+	+
UMAB37	origene	mouse	IgG1	Protein	-	+	+
UMAB38	origene	mouse	IgG1	Protein	-	+	+
UMAB39	origene	mouse	IgG1	Protein	-	+	+
UMAB58	origene	mouse	IgG1	Protein	-	+	+
OTI1H4	origene	mouse	IgG1	Protein	-	+	+
OTI2C11	origene	mouse	IgG2b	Protein	-	+	+
OTI3C4	origene	mouse	IgG1	Protein	-	+	+
OTI4A4	origene	mouse	IgG1	Protein	-	+	+
OTI4B4	origene	mouse	IgG1	Protein	-	+	+
OTI10A5	origene	mouse	IgG1	Protein	-	+	+
OTI11F7	origene	mouse	IgG1	Protein	-	+	+
MEM-97	abcam	mouse	IgG1	Raji cells	+	-	-
B-Ly1	abcam	mouse	IgG1	not available	+	-	-
2H7	abcam	mouse	IgG2b	B cells	+	-	-
B9E9	abcam	mouse	IgG2a	Daudi cells	+	-	-
B-H20	abcam	mouse	IgG2a	CLL cells	+	-	-
GT0008	genetex	mouse	IgG2a		+	-	-
LT20	genetex	mouse	IgG2a	Lymphocytes	+	-	-
ICO-180	Thermo	mouse	IgG1	B cells	+	-	-
743AB35	origene	mouse	IgG2a	cDNA	+	-	-
743AB71	origene	mouse	IgG2a	cDNA	+	-	-
743X45	origene	mouse	IgG2a	cDNA	+	-	-
743X56	origene	mouse	IgG2b	cDNA	+	-	-
743X69	origene	mouse	IgG2a	cDNA	+	-	-
743X78	origene	mouse	IgG2a	cDNA	+	-	-
743X65	origene	mouse	IgG2a	cDNA	+	-	-
IGEL/773	abcam	mouse	IgG2a	Protein	-	+	-
3E9D3C1G3	genetex	mouse	IgG	Peptide	-	+	-
OTI1C12	origene	mouse	IgG1	Protein	-	-	+

i.c., intracellurar

Chapter 2: Establishment of a novel anti-CD20 monoclonal antibody (C_{20}Mab-60) for immunohistochemical analyses

Abstract

There is a requirement for sensitive and specific monoclonal antibodies (mAbs) for detection of CD20, a B-lymphocyte antigen, in every application used for the diagnosis of B-cell lymphoma. Although many anti-CD20 mAbs have been established till date, their applications are limited. The purpose of this study was to establish sensitive and specific anti-CD20 mAbs for use in broad applications, such as flow cytometry, western blot, and immunohistochemical analyses. This study employed the CBIS method, in which all procedures are performed using CD20-stable transfectants, and developed the clone C_{20}Mab-60 (Immunoglobulin [Ig] G_{2a}, kappa). Using flow cytometry, it was found that C_{20}Mab-60 detected overexpression of CD20 in LN229 cell lines and endogenous CD20 in BALL-1 cells (human B cell leukemia cell line), but did not react with CD20-knockout BALL-1 cells (BINDS-24), indicating that C_{20}Mab-60 is specific for CD20. In western blot, C_{20}Mab-60 detected not only the overexpression of CD20 in CHO-K1 cells,

but also the CD20 in BALL-1 cells and Raji cells (human Burkitt's lymphoma cell line), with both high sensitivity and specificity. Furthermore, upon using C_{20}Mab-60 in immunohistochemical analyses, it strongly stained the B-cell lymphomas. These results indicated that C_{20}Mab-60 is useful for the detection of cells expressing CD20 in lymphoma tissues by means of flow cytometry, western blot, and immunohistochemical analyses.

1. Introduction

CD20 is expressed on B cells, from the stages of pre-B to mature B cell development, and is also detected in many types of NHL (21). CD20 is detected in 50% of B-lymphoblastic leukemia/lymphoma originating from pre-B cells, but is not detected in terminally differentiated plasma cell malignancies (22, 23).

CD20 has four membrane-spanning domains and consists of 297 aa, with a molecular weight of 33–37 kDa (19, 20). The two extracellular domains are located at 72–80 aa and 142–182 aa. Although the development of sensitive and specific monoclonal antibodies (mAbs) is critical for the diagnosis of many types of cancer, it is very difficult to detect the extracellular loop of multi-pass transmembrane proteins (18). Because CD20 possesses two extracellular transmembrane loops that are very small in size, it is much more difficult to develop sensitive and specific anti-CD20 mAbs for use in multiple applications, compared to the production of mAbs against single-pass transmembrane proteins, such as CD44 (25) or PD-L1 (9).

As described in the previous chapter, an anti-CD20 antibody, C_{20}Mab-11, was developed using the Cell-Based Immunization Screening (CBIS) method, in which cell

lines are used exclusively for both immunization and screening. The developed mAb was found to be useful for the detection of cells expressing CD20 in lymphoma tissues by means of flow cytometry (FCM), western blot (WB), and immunohistochemical (IHC) analyses. However, the subtype of C_{20}Mab-11 is Immunoglobulin (Ig) M, which is the first antibody to appear in the immune response and has a lower affinity than IgG. In addition, C_{20}Mab-11 was weakly reactive in the IHC against B-cell lymphomas. Therefore, the next step of the study aimed to develop IgG antibodies (with better reactivity) against CD20 using the CBIS method.

2. Materials and Methods

2.1. Cell lines

P3X63Ag8U.1 (P3U1), Chinese hamster ovary (CHO)-K1, Lec1, Lec2, Lec8, and LN229 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Raji (human Burkitt's lymphoma cell line) and BALL-1 (human B cell leukemia cell line) were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan). DNA encoding the CD20 gene (IRAL012D02) was provided by RIKEN BRC through the National BioResource Project of MEXT, Japan. The open reading frame of CD20 plus an N-terminal PA-tag (26) was subcloned into a pCAG-Neo or pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). CHO/CD20 was produced by transfecting pCAG-Neo/CD20 into CHO-K1 cells using a Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories, Berkeley, CA, USA). LN229/CD20 cells were transfected with pCAG-Ble/CD20 into LN229 cells using the Neon™ Transfection System (Thermo Fisher Scientific, Waltham, MA, USA). Lec1/CD20 (N-glycan-deficient), Lec2/CD20 (sialic-acid-deficient), and Lec8/CD20 (galactose-deficient) were

produced by transfecting pCAG-Ble/CD20 into Lec1, Lec2, and Lec8 cells, respectively, using the Neon™ Transfection System. The BALL-1/CD20-KO (BINDS-24) cell line was generated by transfecting the cells with CRISPR/Cas9 plasmids for CD20 (Thermo Fisher Scientific) using the Neon™ Transfection System. Stable transfectants were established using the SH800 cell sorter (Sony Corp., Tokyo, Japan).

P3U1, CHO-K1, CHO/CD20, Lec1/CD20, Lec2/CD20, Lec8/CD20, Raji, BALL-1, and BINDS-24 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque Inc., Kyoto, Japan). LN229 and LN229/CD20 cells were cultured in Dulbecco's modified Eagle medium (Nacalai Tesque Inc.). The media were supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific), 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (Nacalai Tesque Inc.). The cells were grown in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.2. Hybridoma production

Two female BALB/c mice (6-week-old) were purchased from CLEA Japan (Tokyo,

Japan). The animals were housed under specific pathogen-free conditions. The animal experiments described for hybridoma production were approved by the Animal Care and Use Committee of Tohoku University (permit number: 2016MdA-153). Briefly, LN229/CD20 cells (1×10^8 cells) were immunized into two BALB/c mice by means of intraperitoneal injection together with Imject Alum (Thermo Fisher Scientific). After three additional immunizations, a booster injection was administered two days before harvesting the spleen cells. Spleen cells were fused with P3U1 cells using polyethylene glycol 1500 (Roche Diagnostics, Indianapolis, IN, USA), following which the hybridomas were grown in RPMI medium supplemented with sodium hypoxanthine, aminopterin, and thymidine (Thermo Fisher Scientific). The culture supernatants were used then subjected to hybridoma screening by means of FCM.

2.3. FCM

Cells were harvested by means of brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque Inc.). After washing with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), the cells were

treated with 1 μg/mL C_{20}Mab-60 for 30 min at 4°C, followed by treatment with Alexa Fluor™ 488-conjugated anti-mouse IgG (1:2000; Cell Signaling Technology, Danvers, MA, USA). Fluorescence data were collected using a Spectral Cell Analyzer SA3800 (Sony Corp.).

2.4. WB analyses

Cell lysates were prepared using 1% Triton™ X-100, following which the cell debris was removed by means of centrifugation. The cell lysates were boiled in sodium dodecyl sulfate sample buffer with a reducing reagent (Nacalai Tesque Inc.). These obtained proteins (10 μg) were electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred onto polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque Inc.), the membranes were incubated with 10 μg/mL of C_{20}Mab-60, 1 μg/mL of NZ-1 (anti-PA-tag), or 1 μg/mL of anti-β-actin (clone AC-15; Sigma-Aldrich, St. Louis, MO, USA), followed by incubation with peroxidase-conjugated anti-mouse IgG secondary antibody (diluted 1:1000; Agilent Technologies, Santa Clara, CA, USA)

or anti-rat IgG (diluted 1:10,000; Sigma-Aldrich) secondary antibody, respectively. Finally, the proteins were detected with ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) using the Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

2.5. IHC analyses

A formalin-fixed paraffin-embedded (FFPE) tissue microarray (catalog number: Z7020072) for lymphomas was purchased from BioChain Institute Inc. (Newark, CA, USA). Tissue sections were autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences Inc., Tokyo, Japan) for 20 min. After blocking with SuperBlock™ T20 (PBS) Blocking Buffer (Thermo Fisher Scientific), the tissue sections were incubated with C_{20}Mab-60 (5 μg/mL) for 1 h at room temperature and treated with the EnVision+ Kit for mouse (Agilent Technologies) for 30 min. Staining was carried out using 3,3'-diaminobenzidine tetrahydrochloride (Agilent Technologies) for 2 min, followed by counterstaining using hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The CD20 peptides synthesized using PEPScreen (Sigma-Aldrich) were immobilized on Nunc MaxiSorp™ 96-well immunoplates (Thermo Fisher Scientific) at a concentration of 1 mg/mL for 30 min at 37°C. After blocking with 0.1% BSA in PBS, the plates were incubated with 1 μg/mL purified C_{20}Mab-60, followed by incubation with 1:2000 dilution of peroxidase-conjugated anti-mouse IgG (Agilent Technologies). The enzymatic reaction was performed using an ELISA POD Substrate TMB Kit (Nacalai Tesque Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories). These reactions were performed at 37°C using a total sample volume of 50–100 mL.

3. Results

3.1. Generation of anti-CD20 mAbs

Two mice were immunized with LN229/CD20 cells. Supernatants from the cultured hybridomas positive for CHO/CD20 and negative for CHO-K1 were selected by means of FCM. Further screening using WB and IHC was also performed for the establishment of C_{20}Mab-60 (IgG_{2a}, kappa).

3.2. FCM analyses

C_{20}Mab-60 reacted with CHO/CD20 cells, but not with CHO cells, upon FCM analysis (Fig. 9). Similarly, C_{20}Mab-60 reacted with LN229/CD20 cells but not with parental LN229 cells. Additionally, FCM was performed using CD20-stable transfectants of glycan-deficient CHO cell lines (Lec1, Lec2, and Lec8), although it was previously reported that CD20 is not glycosylated (30). C_{20}Mab-60 reacted with Lec1/CD20 (N-glycan-deficient), Lec2/CD20 (sialic-acid-deficient), and Lec8/CD20 (galactose-deficient) cells, but not with parental Lec1, Lec2, and Lec8 cells, indicating that the binding epitope of C_{20}Mab-60 is independent of glycans. Furthermore, C_{20}Mab-60

recognized the endogenous CD20 of BALL-1 cells but did not react with CD20-knockout BALL-1 (BINDS-24). These results indicated that C_{20}Mab-60 is specific for CD20.

3.3. WB analyses

Next, WB was performed using C_{20}Mab-60. C_{20}Mab-60 detected CD20, with a 45-kDa band in CHO/CD20 cells, and a 37-kDa band in BALL-1 and Raji cells, while it did not detect CD20 in CHO-K1 cells and CD20-knockout BALL-1 (BIND-24) cells (Fig. 10), indicating that C_{20}Mab-60 is specific for CD20. An anti-PA-tag mAb (NZ-1) detected CD20 at a 45-kDa band and did not detect any bands in CHO-K1, BALL-1, BINDS-24, and Raji cells. The difference in molecular size detected between CHO/CD20 and BALL-1 cells might be due to the N-terminal PA-tag of CD20.

3.4. IHC analyses

The immunohistochemical utility of C_{20}Mab-60 was further investigated in the lymph node of an oropharyngeal squamous cell carcinoma patient. C_{20}Mab-60 strongly stained the lymph follicles (Fig. 11A-B). No staining was observed without the primary antibody

(Fig. 11C-D). HE staining was also performed on consecutive SCC tissues (Fig. 11E-F).

Additionally, IHC was performed using 38 sections of B-cell lymphomas (Table 4). When staining was performed using C_{20}Mab-11, it presented reactions in only two specimens (Fig. 12A). In contrast, staining with C_{20}Mab-60 resulted in reactions in as many sections as 36 specimens (Fig. 12B). These results indicated that C_{20}Mab-60 is useful for detecting B cells in IHC analyses of FFPE tissues.

3.5. Determination of C_{20}Mab-60 epitope using ELISA

To investigate the epitope of C_{20}Mab-60, ELISA was performed using a peptide synthesized from CD20 160-179 aa, which showed strong reactivity (Fig. 13A). As a next step, 20 peptides were prepared, in which each amino acid in the 160-179aa region was replaced with A or G (Table 5), following which their reactivity was tested using ELISA. The results showed that C_{20}Mab-60 showed the same level of reactivity to P160A, Y161A, I162A, N163A, I164A, Y165A, N166A, S167A, E168A, P169A, K175A, N176A, S177A, P178A, and S179A, as did the wild-type sequence. In contrast, C_{20}Mab-60 did not react with N171A, P172A, S173A, and E174A, and reacted only moderately with A170G (Fig.

13A). This indicated that the CD20 sequence Ala170, Asn171, Pro172, Ser173, and Glu174 is a critical epitope of C_{20}Mab-60. The results are summarized in Fig. 13B.

4. Discussion

In this study, a sensitive and specific anti-CD20 mAb, C_{20}Mab-60, was generated using the CBIS method (18). The CBIS method is an easier and more effective method for development of mAbs against multi-pass transmembrane proteins, because it does not require purified proteins for immunization and screening. Rituximab [a mouse-human chimeric mAb; the original mouse clone 2B8 (32)] is the first anti-CD20 mAb approved by the U. S. Food and Drug Administration that is used for the treatment of B-cell NHL or B-cell chronic lymphocytic leukemia (33). Rituximab shows high sensitivity in FCM, as well as, high antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and anti-tumor activities. Although rituximab could not detect CD20 protein by means of WB in my study (data not shown), C_{20}Mab-60 could detect endogenous CD20 protein, which is expressed in BALL-1 and Raji cells (Fig. 10). C_{20}Mab-60 also stained B-cell lymphoma cells in IHC analyses of FFPE tissues (Fig. 12B). In future studies, it would be useful to further validate my results in more tissues with different degrees of disease progression.

5. Figures and tables

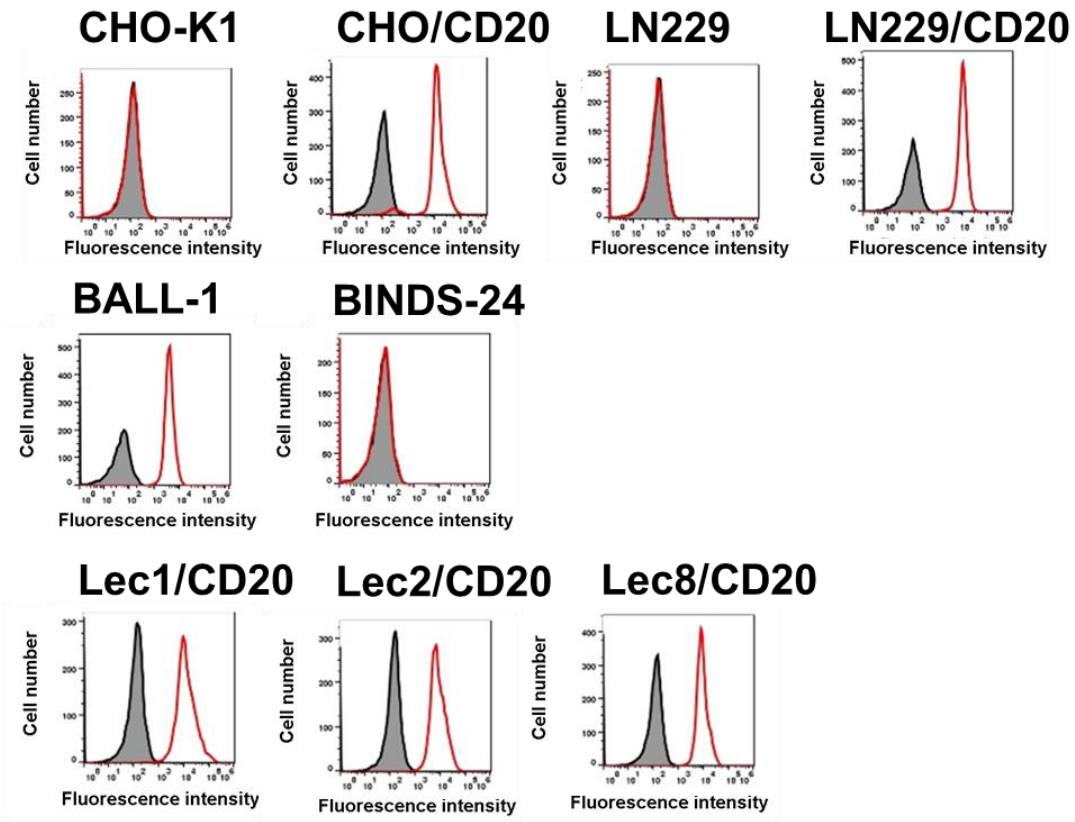


Fig. 9 Detection of CD20 by C_{20}Mab-60 using flow cytometry

CHO-K1, CHO/CD20, LN229, LN229/CD20, Lec1 (N-glycan-deficient), Lec1/CD20, Lec2 (sialic acid-deficient), Lec2/CD20, and Lec8 (galactose-deficient), Lec8/CD20, BALL-1, and BINDS-24 (CD20-deficient BALL-1) cells were treated with C_{20}Mab-60 (red line) at a concentration of 1 μg/mL or with 0.1% BSA in PBS (gray) for 30 min, followed by incubation with secondary antibodies.

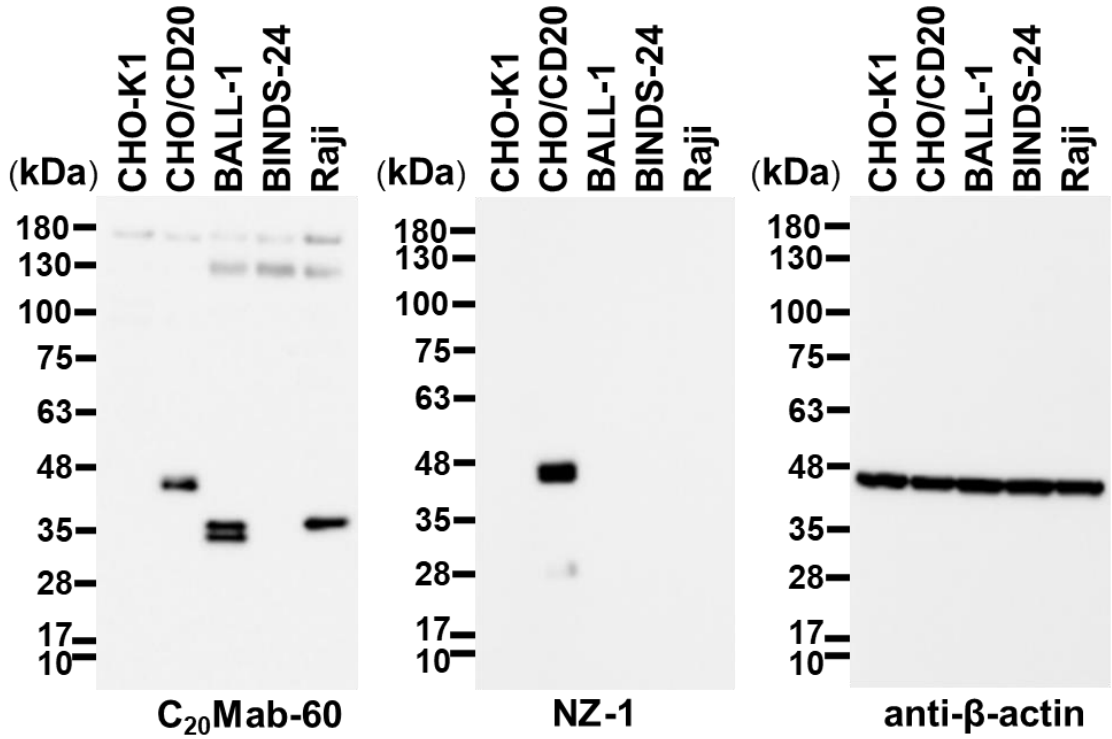


Fig. 10 Western blot using C_{20}Mab-60

Detection of CD20 by C_{20}Mab-60 using western blot. Lysates of CHO-K1, CHO/CD20, BALL-1, BINDS-24, and Raji cells were electrophoresed and transferred onto PVDF membranes. These membranes were then treated with C_{20}Mab-60 (left panel), NZ-1 (anti-PA-tag; middle panel), or anti-β-actin (right panel), followed by incubation with secondary antibodies.

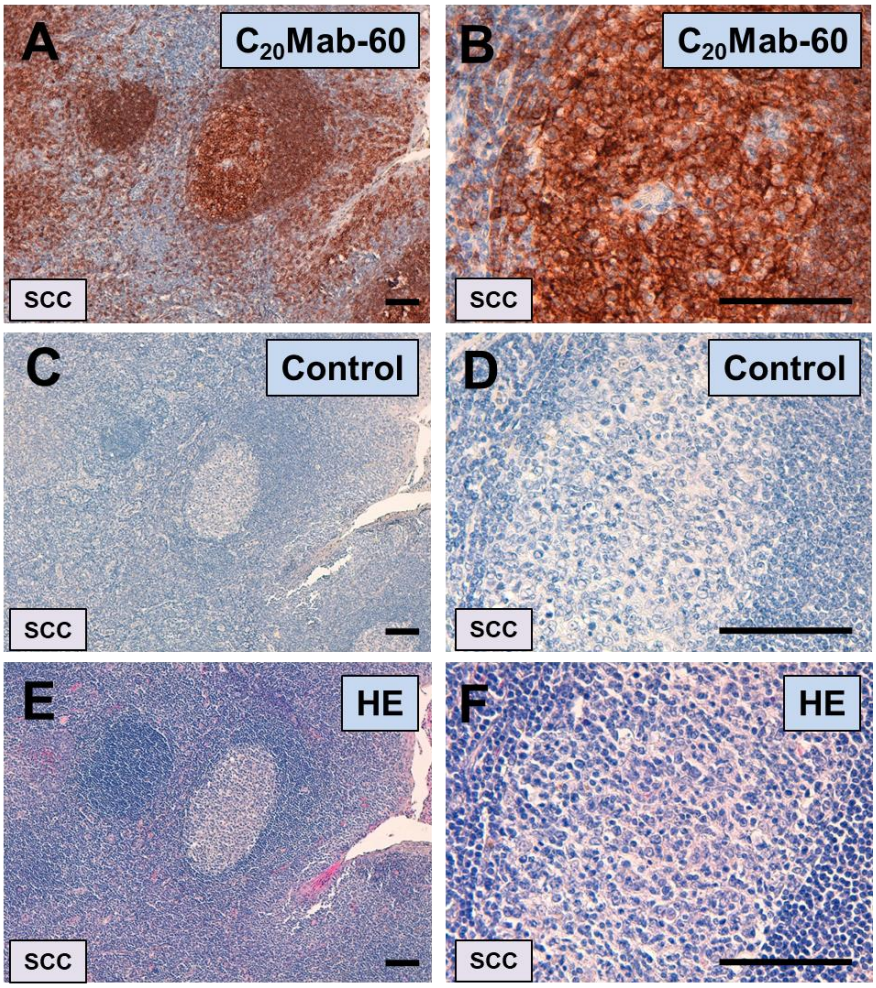
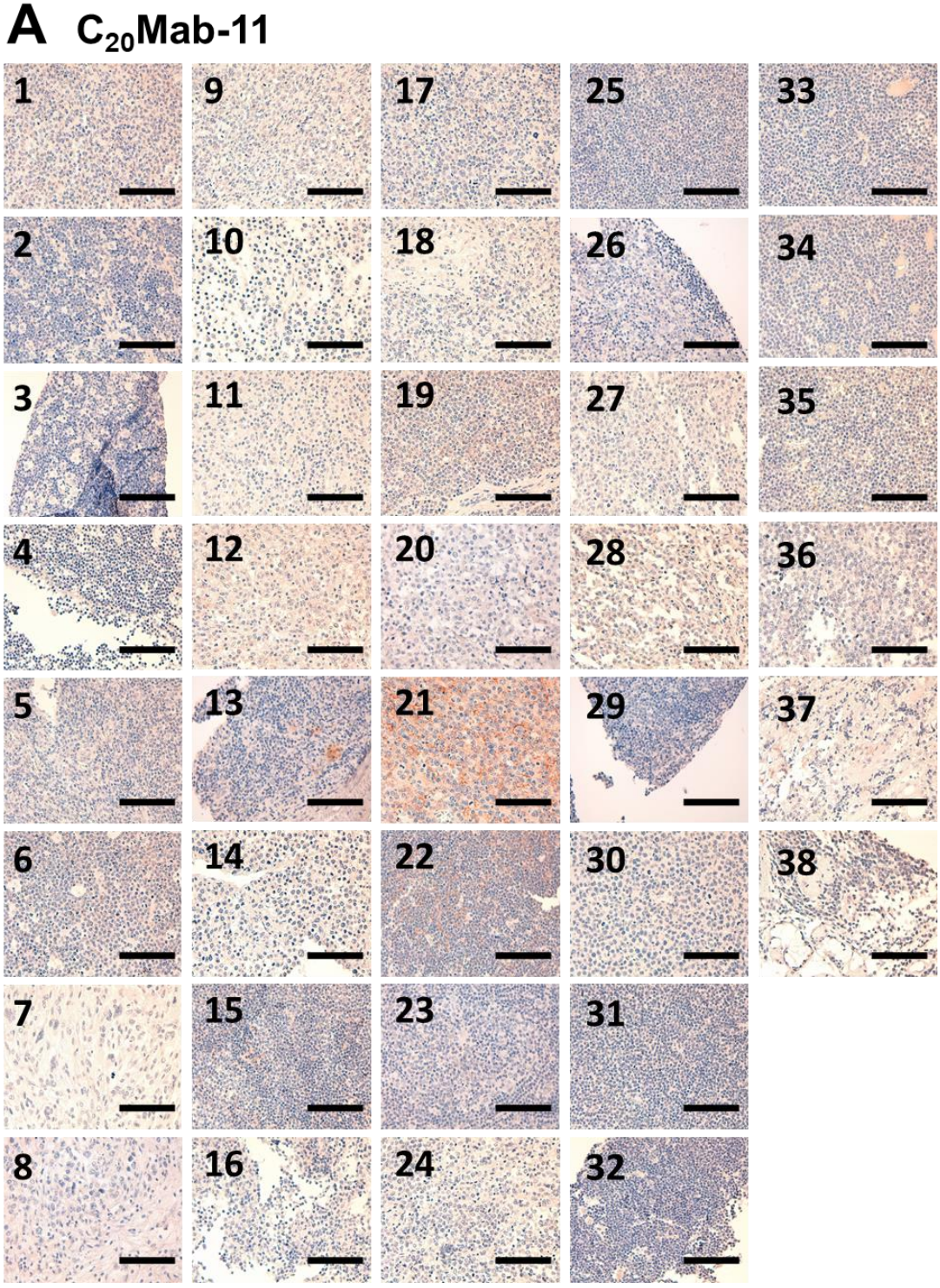


Fig. 11 Immunohistochemical analyses using C_{20}Mab-60 for oropharyngeal squamous cell carcinoma

Consecutive tissue sections of oropharyngeal squamous cell carcinoma were incubated with C_{20}Mab-60 (A-B) or blocking buffer (C-D), followed by treatment with EnVision+ Kit. Counterstaining was performed using hematoxylin (A-D). Hematoxylin and eosin (HE) staining was also performed on consecutive SCC tissues (E-F). Scale bar: 100 μm.


A C20Mab-11
1
9
17
25
33
2
10
18
26
34
3
11
19
27
35
4
12
20
28
36
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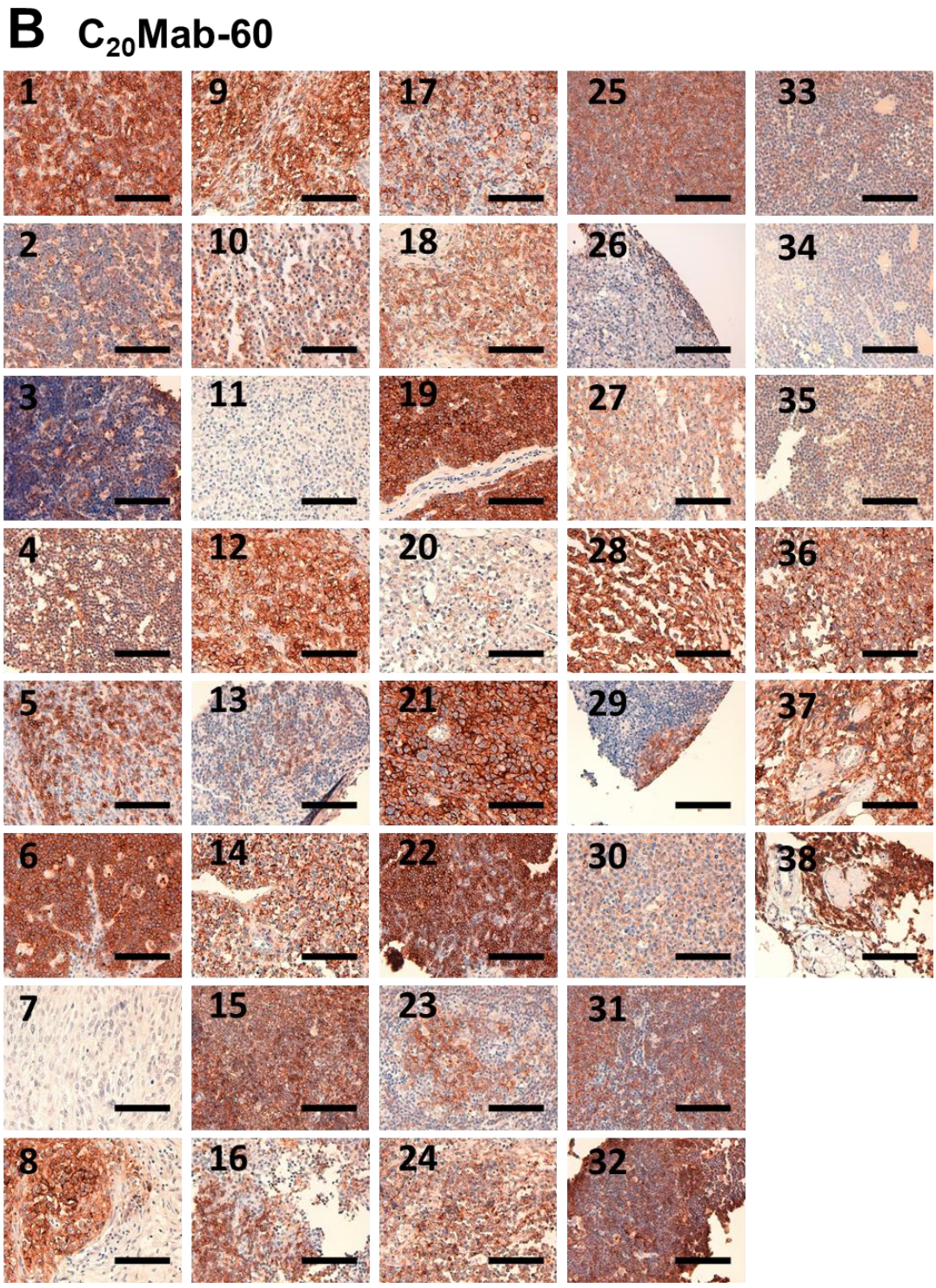

Fig. 12 Immunohistochemical analyses using C_{20}Mab-11 and C_{20}Mab-60 for B-cell lymphomas

Consecutive tissue sections of B-cell lymphomas were incubated with C_{20}Mab-11 (A) or C_{20}Mab-60 (B), followed by treatment with EnVision+ Kit. Scale bar: 100 μm.

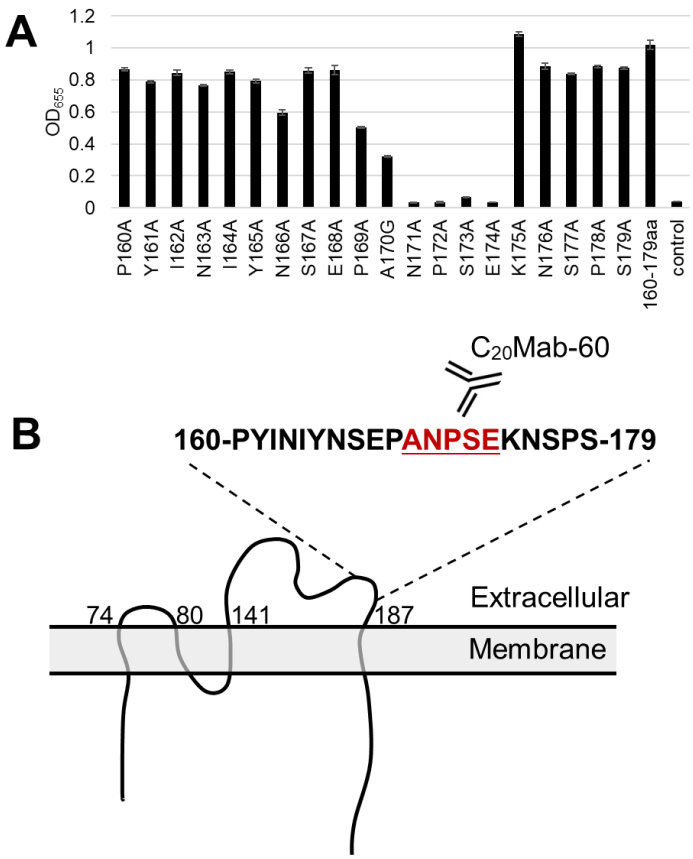

Fig. 13 Determination of the C_{20}Mab-60 epitope for CD20 using ELISA with point mutants

(A) Synthesized peptides of CD20 were immobilized on immunoplates. The plates were then incubated with C_{20}Mab-60 (1 μg/mL), followed by peroxidase-conjugated anti-mouse immunoglobulins. (B) Schematic illustration of CD20 and the C_{20}Mab-60 epitope. The C_{20}Mab-60 epitope of CD20 involves Ala170, Asn171, Pro172, Ser173, and Glu174.

Table 4. Immunohistochemical analyses using C_{20}Mab-11 and C_{20}Mab-60 for B-cell lymphomas.

number	Sex	Age	Site	Histology	C20Mab-11	C20Mab-60
1	F	77	Tyroid	MALT-B cell lymphoma, high grade	—	+
2	F	24	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
3	F	24	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
4	M	55	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
5	M	54	Shoulder	non-Hodgkin B cell lymphoma	—	+
6	M	39	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
7	M	52	Leg	non-Hodgkin B cell lymphoma	—	—
8	F	27	Lymph node	diffuse large B cell lymphoma	—	+
9	M	48	Intestine, colon	non-Hodgkin lymphoma	—	+
10	M	60	Intestine, colon	non-Hodgkin B cell lymphoma	—	+
11	M	72	Peritoneal cavity	non-Hodgkin B cell lymphoma	—	—
12	M	42	Lymph node, axillary	non-Hodgkin B cell lymphoma	—	+
13	M	7	Lymph node, neck	Hodgkin lymphoma	—	+
14	M	66	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
15	M	33	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
16	M	51	Lymph node, neck	anaplastic large Bcell lymphoma	—	+
17	F	32	Lymph node, lung	non-Hodgkin B cell lymphoma	—	+
18	M	72	Testis	non-Hodgkin B cell lymphoma	—	+
19	F	71	Lymph node	non-Hodgkin B cell lymphoma	—	+
20	M	43	Stomach	MALT-B cell lymphoma, high grade	—	+
21	F	52	Lymph node, neck	diffuse large B cell lymphoma	+	+
22	M	60	Intestine, colon	non-Hodgkin B cell lymphoma	+	+
23	M	30	Lymph node, neck	Hodgkin lymphoma	—	+
24	F	33	Stomach	MALT-B cell lymphoma, high grade	—	+
25	F	70	Lymph node, mesentery	non-Hodgkin B cell lymphoma	—	+
26	F	43	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
27	M	76	Tonsil	non-Hodgkin B cell lymphoma	—	+
28	M	65	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
29	M	39	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
30	M	53	Lymph node, neck	diffuse large B cell lymphoma	—	+
31	M	64	Lymph node, neck	non-Hodgkin B cell lymphoma	—	+
32	F	58	Lymph node	non-Hodgkin B cell lymphoma	—	+
33	M	45	Pelvic cavity	mantle cell lymphoma	—	+
34	M	45	Pelvic cavity	mantle cell lymphoma	—	—
35	M	45	Pelvic cavity	mantle cell lymphoma	—	+
36	F	52	Lymph node	mantle cell lymphoma	—	+
37	M	69	Lymph node, neck	mantle cell lymphoma	—	+
38	M	69	Lymph node, neck	mantle cell lymphoma	—	+
					2/38	35/38

Table 5. Identification of C_{20}Mab-60 epitope using synthesized peptides by ELISA.

Peptides	*Sequence*	*C_{20}Mab-60*
P160A	**A**YINIYNSEPANPSEKNSPS	+++
Y161A	P**A**INIYNSEPANPSEKNSPS	+++
I162A	PY**A**NIYNSEPANPSEKNSPS	+++
N163A	PYI**A**IYNSEPANPSEKNSPS	+++
I164A	PYIN**A**YNSEPANPSEKNSPS	+++
Y165A	PYINI**A**NSEPANPSEKNSPS	+++
N166A	PYINIY**A**SEPANPSEKNSPS	+++
S167A	PYINIYN**A**EPANPSEKNSPS	+++
E168A	PYINIYNS**A**PANPSEKNSPS	+++
P169A	PYINIYNSE**A**ANPSEKNSPS	+++
A170G	PYINIYNSEP**G**NPSEKNSPS	++
N171A	PYINIYNSEPA**A**PSEKNSPS	-
P172A	PYINIYNSEPAN**A**SEKNSPS	-
S173A	PYINIYNSEPANP**A**EKNSPS	-
E174A	PYINIYNSEPANPS**A**KNSPS	-
K175A	PYINIYNSEPANPSE**A**NSPS	+++
N176A	PYINIYNSEPANPSEK**A**SPS	+++
S177A	PYINIYNSEPANPSEKN**A**PS	+++
P178A	PYINIYNSEPANPSEKNS**A**S	+++
S179A	PYINIYNSEPANPSEKNSP**A**	+++
160-179aa	PYINIYNSEPANPSEKNSPS	+++

Bold ‘‘A’’ or ‘‘G’’ indicates the substitution from original amino acid. +++, $OD_{655} \geqq 0.5$; ++, $0.3 \leqq OD_{655} < 0.5$; +, $0.1 \leqq OD_{655} < 0.3$; -, $OD_{655} < 0.1$.

General discussion

It is difficult to produce antibodies against membrane proteins with complex structures (40). This is because it is difficult to obtain a target protein with an accurate three-dimensional structure that could be used as an antigen (41), and also difficult to efficiently screen for high-quality antibodies thereafter. This study investigated the possibility of using the CBIS method to easily produce antibodies against membrane proteins. Generally, producing antibodies against membrane proteins, such as CD20, is difficult because it has multiple transmembrane domains, and a small extracellular region. In the present study, as a result of using the CBIS method, it was possible to produce a large number of anti-CD20 antibodies, and many of the antibodies obtained were found to be multi-use antibodies, where a single antibody itself could be used for applications in FCM, WB, and IHC. In general, there are a limited number of applications that a single antibody can be used for (31). This makes it necessary to use different antibodies for different experiments, resulting in incorrect results due to differences in the epitopes and reactivity of the antibodies (42). However, the use of multi-use antibodies obtained using the CBIS method can avoid such problems. In addition, when producing therapeutic antibodies, it

is very important that they react only with the target molecules. Therefore, it is necessary to evaluate their reactivity against various cells. In such cases, multi-use antibodies can be tested on tissue sections using IHC, to easily screen the best antibodies for therapeutic use.

This study resulted in the development of two sensitive and specific anti-CD20 mAbs, C_{20}Mab-11 and C_{20}Mab-60. These antibodies are useful not only for FCM applications, but also WB and IHC. In particular, C_{20}Mab-60 can detect CD20 with high sensitivity in IHC. Upon analyzing 38 lymphoma FFPE tissue samples using IHC, C_{20}Mab-11 reacted with only two samples, while C_{20}Mab-60 reacted with 35 samples. This suggests that C_{20}Mab-60 is particularly useful for the analysis of CD20 using IHC. The epitope of C_{20}Mab-11 recognizes a much broader area of CD20 than that of C_{20}Mab-60, which is likely to be affected by the denaturation of the protein conformation, resulting in reduced reactivity in IHC. In addition to differences in epitopes, affinity and tissue infiltration by antibody subtypes may also have an effect on their recognition of CD20.

It is known that many anti-CD20 antibodies for the treatment of B-cell

lymphoma recognize the 168-EPANPSEKN-176 epitope of CD20, and that the core epitope of rituximab is 170-ANPS-173 (43). However, there are no antibodies that can be used for IHC that recognize the extracellular region of CD20. Therefore, to detect CD20 using IHC, it is necessary to use antibodies such as L26 that recognize the intracellular region of CD20 (44). However, mutations in the epitopes of rituximab (45) and L26 (46) are known to occur in B-cell lymphoma, which leads to a problem of inconsistency between therapeutic efficacy and diagnostic results. On the other hand, the epitope of C_{20}Mab-60 is 170-ANPSE-174, which implies that it recognizes a region very similar to rituximab. Thus, the use of C_{20}Mab-60 may enable the diagnosis of lymphoma using IHC, which may even reflect the response to treatment.

CD20, the target of the antibodies developed in this study, is a multiple transmembrane protein that is considered to be a difficult antigen to produce antibodies against, due to its small extracellular region. There is no single commercially available antibody against CD20 that can be used in all the applications of FCM, WB, or IHC. This reflects the difficulty of producing antibodies against CD20. In this study, the positive rate of FCM against CD20 in the first screening was 3.4% (66/1920 well), which was

very low. This may be because the extracellular region of CD20 is very small and the amino acid sequences of human CD20 and mouse CD20 are similar (identity is 66%, similarity is 94%), resulting in low antigenicity. In contrast, 11 out of 36 clones of the established monoclonal antibodies were found to be usable for FCM, WB, and IHC (data not shown). This suggests that the CBIS method can be used to efficiently produce multi-use antibodies, even for multiple transmembrane proteins such as CD20.

In antibody production using the CBIS method, the cells are directly administered to the animals, and by doing so, the antigens retain their glycans. Therefore, it is possible to obtain antibodies called GpMab that simultaneously recognize the glycans and peptides of the target protein (47). Such antibodies can recognize large three-dimensional structures, and thus, have higher specificity. Currently, there is no easy method for analyzing glycans (48). Therefore, such antibodies are considered very useful in glycan research. In addition, abnormal glycosylation occurs in cancer cells, which differs from that in normal cells (49). When cancer cells are used to express antigens in the CBIS method, antibodies known as cancer-specific monoclonal antibodies (CasMabs), which simultaneously recognize cancer-specific glycans and peptides of such target

proteins can be obtained (50). Using CasMabs, it is possible to specifically detect only the target protein expressed in cancer cells, even when the target protein is also expressed in normal cells. In such a way, it is possible to create new antibody drugs without any side effects.

In this study, I tried to obtain GpMabs or CasMabs against CD20, but these antibodies could not be obtained. It has been reported that glycosylation is not observed in CD20 (51). The possibility of abnormal glycosylation was also explored in this study by expressing CD20 in cancer cells. In this study, I attempted to confirm whether the developed antibodies recognize glycans, but were unable to obtain such antibodies. This may be due to the fact that the glycans are not added to CD20 or that there is very low probability of glycans being added to CD20. Therefore, in my future studies, I would like to develop CasMabs against target proteins such as podoplanin that is known to be highly glycosylated protein.

In conclusion, the results of this thesis indicate that the CBIS method can efficiently produce multi-use antibodies against multiple transmembrane proteins. This suggests that the CBIS method is useful for producing antibodies against proteins with

complex structures, such as G-protein-coupled receptors. In addition, the CBIS method can be used to immunize cells expressing multiple target proteins. Therefore, it may be possible to produce antibodies that simultaneously recognize two proteins that form a complex on the cell membrane. This would make it possible to create new therapeutic antibodies that recognize specific combinations of proteins, such as integrins. In addition, the binding of such antibodies can stabilize the two proteins into a state of normal interaction. This may be useful for conformational analyses. For the above reasons, the CBIS method can produce unprecedented antibodies, which will boost research in Biochemistry and Medicine.

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