MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 39, Number 4, 2020

DOI: 10.1089/mab.2020.0015

Establishment of an Anti-CD20 Monoclonal Antibody (C_{20}Mab-60) for Immunohistochemical Analyses

Yoshikazu Furusawa,[1,2] Mika K. Kaneko,[1] and Yukinari Kato[1,2]

Sensitive and specific monoclonal antibodies (mAbs) are needed for detecting CD20. This antigen, one of several B lymphocyte antigens, is a target for every application used in the diagnosis of B cell lymphoma. Many anti-CD20 mAbs have been established, although applications of these antibodies are limited. This study aims to establish sensitive and specific anti-CD20 mAbs suitable for broad application, such as flow cytometry, Western blotting, and immunohistochemical analyses. Using the Cell-Based Immunization and Screening (CBIS) method, all procedures were performed by utilizing CD20-stable transfectants, and a clone, C_{20}Mab-60 (IgG_{2a}, kappa), was developed. In flow cytometry, C_{20}Mab-60 detected overexpression of CD20 in LN229 cell and endogenous CD20 in BALL-1 (a human B cell leukemia cell line) but did not react with CD20-knockout BALL-1 (BINDS-24), indicating specificity for CD20. In Western blotting, C_{20}Mab-60 detected CD20-overexpressing Chinese hamster ovary-K1, BALL-1, and Raji (a human Burkitt's lymphoma cell line) displaying both sensitivity and specificity. Furthermore, B cell but not T cell lymphomas were strongly stained with C_{20}Mab-60 in immunohistochemical analyses. C_{20}Mab-60, which was developed by CBIS method, is shown to be useful for the detection of cells expressing CD20 in lymphoma tissues by flow cytometry, Western blotting, and immunohistochemical analyses.

Keywords: CD20, monoclonal antibody, Western blotting, flow cytometry, immunohistochemistry

Introduction

CD20 is expressed in B cells from pre- to mature phases of cell development and is also detected in many types of non-Hodgkin lymphoma (NHL).[1] CD20 is detected in 50% of B-lymphoblastic leukemia/lymphomas (B-ALL/LBL), originating from pre-B cells; however, it is not detected in terminally differentiated plasma cell malignancies.[2,3]

CD20 has four membrane-spanning domains, consisting of 297 amino acids (aa), with a molecular weight of 33–37 kDa.[4,5] The two extracellular domains are located at 72–80 and 142–182 aa. Sensitive and specific monoclonal antibodies (mAbs) are critical for the diagnosis of many types of cancer, but the detection of extracellular loops of multipass transmembrane proteins is quite difficult.[6] CD20 possesses two small extracellular transmembrane loops, increasing the difficulty of developing useful mAbs for use in multiple applications. This difficulty contrasts with the relative ease of production of mAbs against single-pass transmembrane proteins, such as CD44[7] or PD-L1.[8]

We have developed the Cell-Based Immunization and Screening (CBIS) method, in which cell lines are exclusively used for both immunization and screening. The CBIS method has previously been used for developing several mAbs against various proteins, including the five transmembrane protein CD133.[6] Using the CBIS method, we have successfully produced sensitive and specific mAbs useful for not only flow cytometry, but also Western blot and immunohistochemical analyses.[6–16] In this study, we aimed to develop multiuse anti-CD20 mAbs that can be used for flow cytometry, Western blot, and immunohistochemical analyses.

Materials and Methods

Cell lines

P3X63Ag8U.1 (P3U1), Chinese hamster ovary (CHO)-K1, 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 the RIKEN BRC through the National BioResource Project of MEXT, Japan. An open reading frame of CD20, plus an N-terminal PA tag, was subcloned into a pCAG-Neo or pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). CHO/CD20 was produced by

[1]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.
[2]New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.

transfecting pCAG-Neo/CD20 into CHO-K1 cells using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). LN229/CD20 was produced by transfecting pCAG-Ble/CD20 into LN229 cells using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell line BALL-1/CD20-KO (BINDS-24) was generated by transfecting CRISPR/Cas9 plasmids for CD20 (Thermo Fisher Scientific, Inc.) using a Neon transfection system. Stable transfectants were established using SH800 (Sony Corp., Tokyo, Japan).

P3U1, CHO-K1, CHO/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 were cultured using Dulbecco's Modified Eagle's Medium (Nacalai Tesque, Inc.). Media were supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). Cells were grown inside an incubator at 37°C with humidity and 5% CO_2/95% air atmosphere.

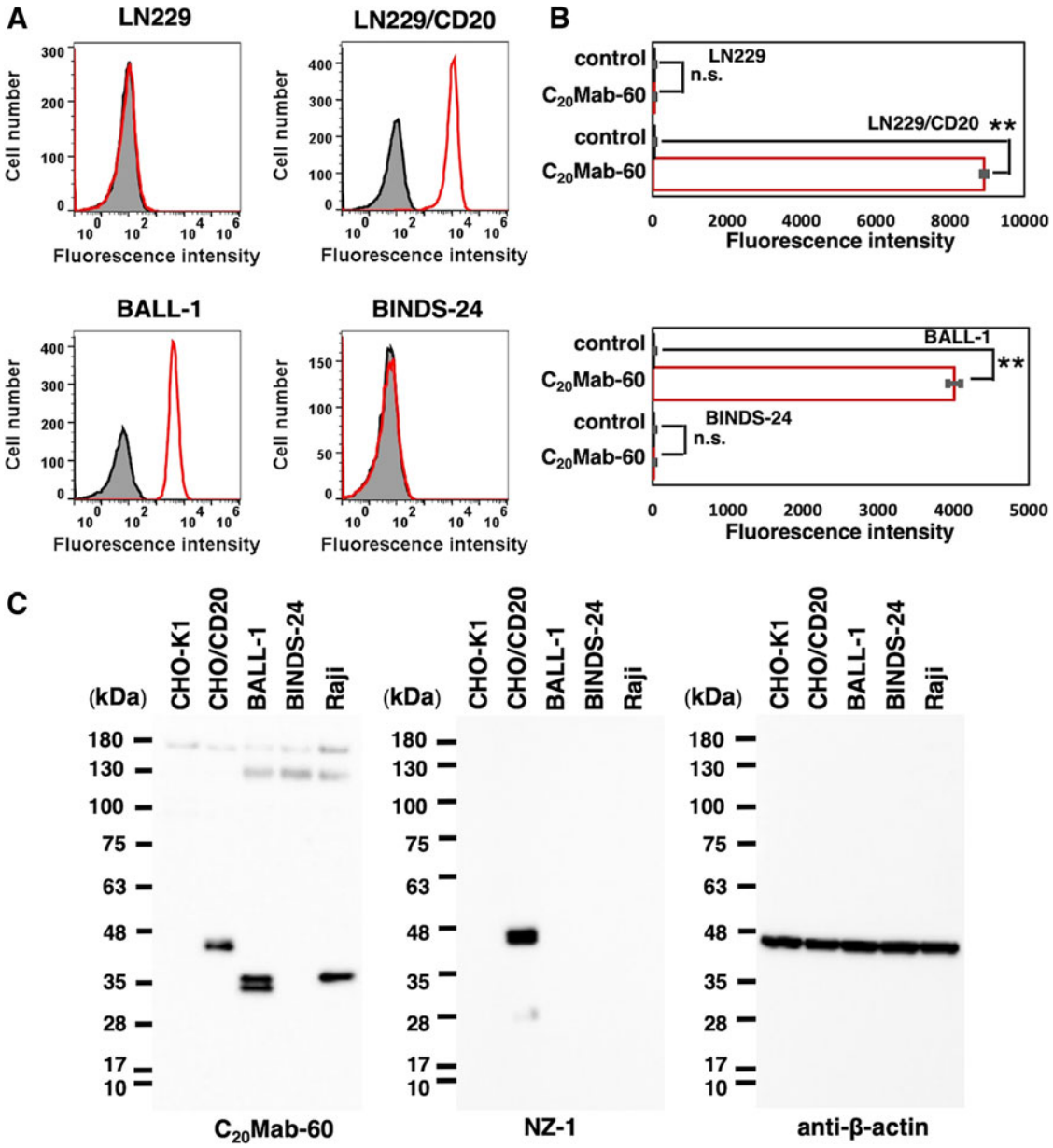

FIG. 1. Detection of CD20 by C_{20}Mab-60 using flow cytometry and Western blotting. **(A)** Detection of CD20 by C_{20}Mab-60. LN229, LN229/CD20, BALL-1, and BIND-24 were treated with C_{20}Mab-60 (red line) at a concentration of 1 μg/mL or 0.1% BSA in PBS (gray) for 30 minutes, followed by incubation with secondary antibodies. **(B)** Fluorescence intensity was quantified. $^{**}p < 0.01$. n.s., not significant. All data are expressed as mean ± SEM. **(C)** Detection of CD20 by C_{20}Mab-60 using Western blotting. Cell lysates of CHO-K1, CHO/CD20, BALL-1, BINDS-24, and Raji cells were electrophoresed and transferred onto PVDF membranes. These membranes were 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. BSA, bovine serum albumin; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; SEM, standard error of the mean.

Hybridoma production

Two female BALB/c mice (6 weeks old) were purchased from CLEA Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions. Animal experiments for hybridoma production were approved by the Animal Care and Use Committee of Tohoku University (Permit No.: 2016MdA-153). In brief, LN229/CD20 cells (1×10^8 cells) were injected intraperitoneally into two BALB/c mice together with Imject Alum (Thermo Fisher Scientific, Inc.). After three additional immunizations, a booster injection was administered 2 days before harvesting spleen cells. Spleen cells were fused with P3U1 cells using polyethylene glycol 1500 (Roche Diagnostics, Indianapolis, IN, USA). The resulting hybridomas were grown in RPMI medium supplemented with hypoxanthine, aminopterin, and thymidine (Thermo Fisher Scientific, Inc.). Culture supernatants were utilized for hybridoma screening by flow cytometry.

Flow cytometry

Cells were harvested by brief exposure to 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, cells were treated with 1 μg/mL of C_{20}Mab-60 for 30 minutes at 4°C, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA, USA). Fluorescence intensity data were collected using Spectral Cell Analyzer SA3800 (Sony Corp.).

Western blotting analyses

Cells were lysed in 1% Triton X-100, and cell debris was removed by centrifugation. Cell lysates were boiled in sodium dodecyl sulfate sample buffer with a reducing reagent (Nacalai Tesque, Inc.). Proteins (10 μg) were separated on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and were then 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 for control (clone AC-15; Sigma-Aldrich Corp., St. Louis, MO, USA), followed by incubation with the secondary antibody, peroxidase-conjugated anti-mouse immunoglobulins (1:1000; Agilent Technologies, Inc., Santa Clara, CA, USA), or anti-rat IgG (1:10,000; Sigma-Aldrich Corp.). Finally, proteins were detected with ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) using the Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Immunohistochemical analyses

A formalin-fixed paraffin-embedded (FFPE) tissue microarray (Cat. No.: Z7020072) for lymphomas was purchased from BioChain Institute Inc. (Newark, CA, USA). Tissue sections were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 min. After blocking with the SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), tissue sections were incubated with C_{20}Mab-60 (5 μg/mL) for 1 hour at room temperature and were then treated with a mouse Envision+ Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

Statistical analyses

Statistical analysis used Student's *t*-test in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). $p < 0.05$ was considered statistically significant. All data are expressed as mean ± standard error of the mean.

Results

Establishment of anti-CD20 mAbs

Two mice were immunized with LN229/CD20 cells. Supernatants from cultured hybridomas, positive for CHO/CD20 and negative for CHO-K1, were selected by flow cytometry. Further screening using Western blotting and immunohistochemistry was performed, and C_{20}Mab-60 (IgG_{2a}, kappa) was established.

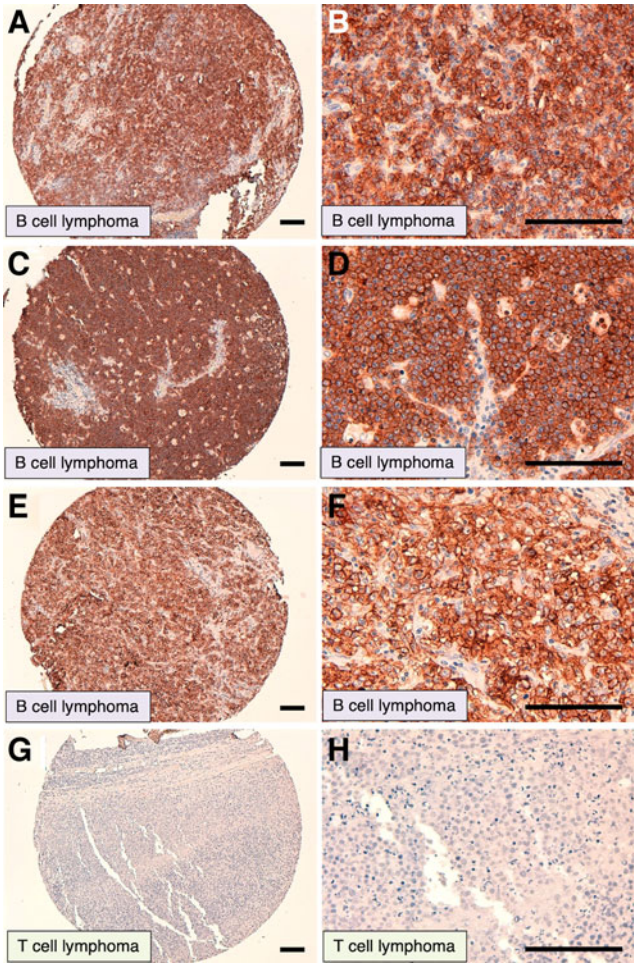

FIG. 2. Immunohistochemical analyses using C_{20}Mab-60 for B cell lymphomas. Tissue sections of B cell **(A–F)** and T cell lymphomas **(G, H)** were incubated with C_{20}Mab-60, followed by an Envision+ kit. **(B, D, F, and H)** are enlargements of **(A, C, E, and G)**, respectively. Counterstaining used hematoxylin. Scale bar = 100 μm.

Flow cytometry

C_{20}Mab-60 reacted with LN229/CD20 cells, but not with LN229 cells, which allowed cells to be distinguished using flow cytometry analysis (Fig. 1A). Furthermore, C_{20}Mab-60 reacted with BALL-1 cells, but not with CD20-knockout BALL-1 cells (BINDS-24), which indicated that C_{20}Mab-60 is specific for CD20 (Fig. 1B).

Western blot analyses

Western blotting detected CD20 using C_{20}Mab-60 in a 45-kDa protein band from CHO/CD20 cells and in a 37-kDa band in BALL-1 and Raji cells. No detection was observed in proteins from CHO-K1 cells and CD20-knockout BALL-1 (BIND-24) cells (Fig. 1C). Thus, 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 this band in proteins from CHO-K1, BALL-1, BINDS-24, and Raji cells. The difference in molecular size of CD20 obtained from CHO/CD20 and BALL-1 cells might be due to the N-terminal PA-tag of CD20 introduced into CHO-K1 cells.

Immunohistochemical analyses

C_{20}Mab-60 was also assessed for its immunohistochemical utility in a B cell lymphoma tissue microarray. C_{20}Mab-60 strongly stained B cell lymphomas (Fig. 2A–F), and no staining was observed in T cell lymphomas (Fig. 2G, H). Thus, C_{20}Mab-60 staining is B cell specific. C_{20}Mab-60 is useful for detecting B cells in immunohistochemical analyses in FFPE-processed tissues.

Discussion

In this study, a sensitive and specific anti-CD20 mAb, C_{20}Mab-60, was developed using the CBIS method.[6] This method is easier and more effective in acquiring mAbs against multipass transmembrane proteins because it has no requirement for purified proteins for immunization and screening. Rituximab (a mouse–human chimeric mAb; the original mouse clone is 2B8[17]) is the first anti-CD20 mAb approved by the US FDA and is currently used to treat B cell NHL and B cell chronic lymphocytic leukemia.[18] Rituximab shows high sensitivity in flow cytometry and exerts high antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, and antitumor activity. Rituximab does not detect CD20 protein in Western blotting (data not shown), but C_{20}Mab-60 does detect endogenous CD20 protein expressed in BALL-1 and Raji cells with high sensitivity (Fig. 1C). C_{20}Mab-60 also stains B cell lymphoma cells in immunohistochemical analyses (Fig. 2). Nonetheless, further study validating the results in supplemental tissues that display different stages of disease progression will provide useful additional characterization of the mAb.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported in part by AMED under Grant Numbers JP20am0401013 (Y.K.), JP20am0101078 (Y.K.), and JP20ae0101028 (Y.K.), and by JSPS KAKENHI Grant Numbers 17K07299 (M.K.K.) and 19K07705 (Y.K.).

References

1. Tedder TF, Boyd AW, Freedman AS, Nadler LM, and Schlossman SF: The B cell surface molecule B1 is functionally linked with B cell activation and differentiation. J Immunol 1985;135:973–979.
2. Rawstron AC, Owen RG, Davies FE, Johnson RJ, Jones RA, Richards SJ, Evans PA, Child JA, Smith GM, Jack AS, and Morgan GJ: Circulating plasma cells in multiple myeloma: Characterization and correlation with disease stage. Br J Haematol 1997;97:46–55.
3. Chu PG, Loera S, Huang Q, and Weiss LM: Lineage determination of CD20- B-Cell neoplasms: An immunohistochemical study. Am J Clin Pathol 2006;126:534–544.
4. Polyak MJ, Li H, Shariat N, and Deans JP: CD20 homooligomers physically associate with the B cell antigen receptor. Dissociation upon receptor engagement and recruitment of phosphoproteins and calmodulin-binding proteins. J Biol Chem 2008;283:18545–18552.
5. Li H, Ayer LM, Lytton J, and Deans JP: Store-operated cation entry mediated by CD20 in membrane rafts. J Biol Chem 2003;278:42427–42434.
6. Itai S, Fujii Y, Nakamura T, Chang YW, Yanaka M, Saidoh N, Handa S, Suzuki H, Harada H, Yamada S, Kaneko MK, and Kato Y: Establishment of CMab-43, a sensitive and specific anti-CD133 monoclonal antibody, for immunohistochemistry. Monoclon Antib Immunodiagn Immunother 2017;36:231–235.
7. Yamada S, Itai S, Nakamura T, Yanaka M, Kaneko MK, and Kato Y: Detection of high CD44 expression in oral cancers using the novel monoclonal antibody, C44Mab-5. Biochem Biophys Rep 2018;14:64–68.
8. Yamada S, Itai S, Nakamura T, Yanaka M, Chang YW, Suzuki H, Kaneko MK, and Kato Y: Monoclonal antibody L1Mab-13 detected human PD-L1 in lung cancers. Monoclon Antib Immunodiagn Immunother 2018;37:110–115.
9. Furusawa Y, Yamada S, Itai S, Sano M, Nakamura T, Yanaka M, Fukui M, Harada H, Mizuno T, Sakai Y, Takasu M, Kaneko MK, and Kato Y: PMab-210: A monoclonal antibody against pig podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38:30–36.
10. Furusawa Y, Kaneko MK, Nakamura T, Itai S, Fukui M, Harada H, Yamada S, and Kato Y: Establishment of a monoclonal antibody PMab-231 for tiger podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38:89–95.
11. Furusawa Y, Takei J, Sayama Y, Yamada S, Kaneko MK, and Kato Y: Development of an anti-bear podoplanin monoclonal antibody PMab-247 for immunohistochemical analysis. Biochem Biophys Rep 2019;18:100644.
12. Furusawa Y, Yamada S, Itai S, Nakamura T, Takei J, Sano M, Harada H, Fukui M, Kaneko MK, and Kato Y: Establishment of a monoclonal antibody PMab-233 for immunohistochemical analysis against Tasmanian devil podoplanin. Biochem Biophys Rep 2019;18:100631.
13. Furusawa Y, Yamada S, Itai S, Nakamura T, Yanaka M, Sano M, Harada H, Fukui M, Kaneko MK, and Kato Y: PMab-219: A monoclonal antibody for the immunohistochemical analysis of horse podoplanin. Biochem Biophys Rep 2019;18:100616.

14. Kato Y, Furusawa Y, Itai S, Takei J, Nakamura T, Sano M, Harada H, Yamada S, and Kaneko MK: Establishment of an anticetacean podoplanin monoclonal antibody PMab-237 for immunohistochemical analysis. Monoclon Antib Immunodiagn Immunother 2019;38:108–113.
15. Kato Y, Furusawa Y, Yamada S, Itai S, Takei J, Sano M, and Kaneko MK: Establishment of a monoclonal antibody PMab-225 against alpaca podoplanin for immunohistochemical analyses. Biochem Biophys Rep 2019;18:100633.
16. Kato Y, Yamada S, Furusawa Y, Itai S, Nakamura T, Yanaka M, Sano M, Harada H, Fukui M, and Kaneko MK: PMab-213: A monoclonal antibody for immunohistochemical analysis against pig podoplanin. Monoclon Antib Immunodiagn Immunother 2019;38:18–24.
17. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, and Anderson DR: Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood 1994;83:435–445.
18. Keating GM: Rituximab: A review of its use in chronic lymphocytic leukaemia, low-grade or follicular lymphoma and diffuse large B-cell lymphoma. Drugs 2010;70:1445–1476.

Address correspondence to:
Yukinari Kato
New Industry Creation Hatchery Center
Tohoku University
2-1, Seiryo-machi, Aoba-ku
Sendai
Miyagi 980-8575
Japan

E-mail: yukinarikato@med.tohoku.ac.jp

Received: March 19, 2020
Accepted: June 17, 2020