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# *De novo* diffuse large B-cell lymphoma with a CD20 immunohistochemistry-positive and flow cytometry-negative phenotype: Molecular mechanisms and correlation with rituximab sensitivity

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**Key words**

Diffuse large B-cell lymphoma, CD20, rituximab, immunohistochemistry, flow cytometry

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**CD20 is expressed in most B-cell lymphomas and is a critical molecular target of rituximab. Some B-cell lymphomas show aberrant CD20 expression, and rituximab use in these patients is controversial. Here we show both the molecular mechanisms and the clinical significance of *de novo* diffuse large B-cell lymphomas (DLBCL) that show a CD20 immunohistochemistry (IHC)-positive and flow cytometry (FCM)- negative (IHC[+]/FCM[−]) phenotype. Both IHC and FCM using anti-CD20 antibodies L26 and B1, respectively, were analyzed in 37 of the 106 cases of *de novo* DLBCL; 8 (22%) of these cases were CD79a(+)/CD20(+) with IHC and CD19(+)/CD20(−) with FCM. *CD20* (*MS4A1*) mRNA expression was significantly lower in IHC(+)/FCM(−) cells than in IHC(+)/FCM(+) cells ($P = 0.0005$). No genetic mutations were detected in *MS4A1* promoter and coding regions. Rituximab-mediated cytotoxicity in the CDC assay using IHC(+)/FCM(−) primary cells was significantly lower than in IHC(+)/FCM(+) cells ($P < 0.05$); however, partial effectiveness was confirmed. FCM using rituximab detected CD20 more efficiently than B1. No significant difference was observed between IHC(+)/FCM(−) and IHC(+)/FCM(+) patients in overall survival ($P = 0.664$). Thus, lower expression of *CD20* mRNA is critical for the CD20 IHC(+)/FCM(−) phenotype. Lower CD20 expression with FCM does not rule out rituximab use in these patients if expression is confirmed with IHC. FCM using rituximab may be more informative than B1 for predicting rituximab effectiveness in IHC(+)/FCM(−) cases.**

CD20 is a cell surface antigen expressed specifically on most human B cells.[1] Because CD20 is also expressed on more than 90% of B-cell lymphoma cells, CD20 has become a good molecular target for monoclonal antibody therapeutics.[2,3] Rituximab is a mouse–human chimeric monoclonal antibody targeting CD20. Previous reports indicate that clinical outcomes in patients with B-cell lymphomas have been significantly improved with rituximab with conventional chemotherapies.[4–6] However, the overall survival (OS) is still not satisfactory because more than 50% of B-cell lymphoma patients show relapse/recurrence of disease after several years.[4] Thus, we believe that confirming the mechanisms of rituximab resistance[7,8] is important for further improving the OS and progression free survival (PFS) of B-cell lymphoma patients.

Recently we reported that downregulation of CD20 protein expression after combination chemotherapy with rituximab is a critical reason for rituximab resistance.[9–11] Other groups have indicated that abnormalities in CD20 expression because of shaving,[12,13] genetic mutations or deletions,[14–16] aberrant splicing,[17] and internalization into the cytoplasm[18,19] strongly correlate with lower sensitivity to rituximab treatment. Furthermore, lower expression of CD20 has been confirmed in even among patients with the same disease, such as diffuse large B-cell lymphoma (DLBCL).[20–22] Previous reports regarding ADCC and CDC activity induced by rituximab indicate that lower protein expression is strongly correlated with the efficacy of anti-CD20 antibodies.[23,24] Thus, knowing the level of CD20 protein expression may be very important in the clinical setting for predicting the outcome of anti-CD20 antibody therapy.

Although we and others recently recognized that some B-cell lymphoma patients show discrepancies in CD20

protein expression showing an immunohistochemistry (IHC)-positive and flow cytometry (FCM)- negative (IHC[+] and FCM[−]) phenotype,[21,25] neither molecular mechanisms of this phenotype nor rituximab sensitivities have been elucidated. In this study, we analyzed the frequency of occurrence and clinical features of *de novo* DLBCL patients who showed the CD20 IHC(+)/FCM(−) phenotype and analyzed the molecular basis of the phenotype using primary clinical samples. In the present study we also examine the rituximab sensitivity of those cells compared with CD20 IHC(+)/FCM (+) B-cell lymphoma cells to determine whether rituximab can still be utilized in those patients in combination with conventional chemotherapies.

## Materials and Methods

**Patients and lymphoma tissue samples.** Between January 2006 and May 2012 in Nagoya University Hospital, 106 patients were diagnosed with *de novo* DLBCL (Table 1). All patients were treated with combination chemotherapy that included rituximab. The final follow up was on 22 November 2012. Lymphoma tissue was harvested and used for pathological analysis, and if a sufficient volume of tissue was obtained, FCM, chromosomal analysis, DNA, RNA and protein extraction, and cryopreservation were performed. Lymphoma tissues showing the CD20 IHC(+)/FCM(−) phenotype in the affiliated hospital were also

**Table 1. Patients' characteristics of DLBCL with CD20 IHC(+)/FCM(−) phenotype**

| | Total | CD20 IHC(+)/FCM(+)‡ | CD20 IHC(+)/FCM(−)§ | *P*-value‡ *vs*§ |
|---|---|---|---|---|
| Patients number (%) | 106 (100) | 29/37 (78) | 8/37 (22) | |
| Age | | | | |
| Median [range] | 66 [26–88] | 65 [35–81] | 60 [52–77] | 0.394 |
| >60 y.o. | 80 (75) | 19 (66) | 3 (38) | 0.228 |
| Gender: male | 74 (70) | 20 (69) | 5 (63) | 1 |
| PS, >1 | 18 (17) | 7 (24) | 0 (0) | 0.308 |
| LDH, >UNL | 61 (58) | 17 (59) | 6 (75) | 0.683 |
| Extra nodal site(s), >1 | 23 (22) | 8 (28) | 2 (25) | 1 |
| Stage, III/IV | 57 (54) | 18 (62) | 6 (75) | 0.685 |
| IPI score at diagnosis | | | | |
| 0, 1 | 33 (31) | 9 (31) | 2 (25) | 0.779 |
| 2 | 35 (33) | 8 (28) | 3 (38) | |
| 3 | 18 (17) | 4 (14) | 2 (25) | |
| 4, 5 | 20 (19) | 8 (28) | 1 (13) | |
| IHC classification† | | | | |
| GCB | 34/72 (47) | 9/23 (39) | 3/5 (60) | 0.624 |
| Non-GCB | 38/72 (53) | 14/23 (61) | 2/5 (40) | |
| EBV status† | | | | |
| EBER-ISH | 6/75 (8) | 0/22 (0) | 0/6 (0) | |
| Light chain restriction in FCM† | | | | |
| Kappa | 13/28 (46) | 9/20 (45) | 4/8 (50) | 0.167 |
| Lambda | 6/28 (21) | 6/20 (30) | 0/8 (0) | |
| Negative | 9/28 (32) | 5/20 (25) | 4/8 (50) | |

†The total patients' number examined in each analysis are indicated as denominators. ‡CD20 IHC(+)/FCM(+). §CD20 IHC(+)/FCM(−). EBV, Epstein–Barr virus; EBER-ISH, EBV-encoded RNA-in situ hybridization; GCB, germinal center B-cell type; IPI, international prognostic index; PS, performance status.

**Table 2. Clinical characteristics and the molecular back grounds of the CD20 IHC(+)/FCM(−) patients**

| UPN | Age | Gender | Diag. | Stage | Patho. source | CD20 expression | | | CDS mutation | Promoter region mutation# | Karyotype | Light chain restriction | IPI (score) | Treatment | Response (alive or death) | Survival |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | IHC | FCM | RT | | | | | | | | |
| 1 | 55 | F | DLBCL | IAE | LN | + | − | → | − | − | Complex | Kappa | Low (0) | R-CHOP | CR (A) | 37M |
| 2 | 60 | M | DLBCL | IVB | LN | + | − | NT | NT | NT | Complex | − | High (4) | R-COP | CR (A) | 37M |
| 3 | 71 | M | DLBCL | IIA | LN | + | − | → | − | − | Complex | Lambda | Low (1) | R-THP-COP | CR (A) | 32M |
| 4 | 66 | M | DLBCL | IIA | LN | + | − | → | − | − | NE | Kappa | Low (1) | R-CHOP | CR (A) | 32M |
| 5 | 92 | F | DLBCL | IIA | LN | + | − | → | − | − | Normal | Kappa | Low (1) | THP-COP | CR (D) | 11M |
| 6 | 51 | M | DLBCL | IA | LN | + | − | → | − | − | NE | Kappa | Low (0) | R-CHOP | CR (A) | 25M |
| 7 | 66 | M | DLBCL | IIIA | LN | + | − | NT | NT | NT | Complex | Kappa | H-I (3) | THP-COP | PD (D) | 48M |
| 8 | 77 | F | DLBCL | IVB | GI, BM | + | − | → | − | − | Normal | − | High (4) | R-CHOP | CR (D) | 13M |
| 9 | 52 | M | DLBCL | IVA | LN | + | − | → | − | − | NE | − | L-I (2) | R-CHOP | CR (A) | 14M |
| 10 | 60 | F | DLBCL | IIIAE | LN | + | − | NT | NT | NT | NE | Kappa | L-I (2) | R-CHOP | CR (A) | 10M |
| 11 | 83 | M | DLBCL | IIIA | LN | + | − | → | − | − | NE | − | H-I (3) | R-EPOCH | NA (A) | 9M |
| 12 | 67 | M | DLBCL | IIA | LN | + | − | → | − | − | Normal | Kappa | L-I (2) | R-CHOP | CR (A) | 7M |

Black arrow, downregulated; BM, bone marrow; CDS, coding sequence of *MS4A1* gene; Diag., diagnosis; GI, gastrointestinal; H-I, high-intermediate; L-I, low-intermediate; LN, lymphnode; NE, not evaluated; NT, not tested; Patho. Source, sources of tumor tissues for pathological analysis; R-CHOP, rituximab, cyclophosphamide, doxorubicin vincristine and prednisolone; RT, RT-PCR; THP, tetrahydropyranyl adriamycin; EPOCH, etoposide, vincristine, cyclophosphamide and prednisolone; #, 1000 bp upstream from the transcription start site (−1000 to +1) of *MS4A1* gene.

sent to our laboratory as snap-frozen samples and utilized. These studies were conducted with institutional review board approval from the Nagoya University School of Medicine, and written informed consent was obtained from each patient analyzed in accordance with the Declaration of Helsinki.

**Primary B-cell lymphoma cells and cell lines.** Primary B-cell lymphoma tissues were separated into single-cell suspensions in 10-cm culture dishes with RPMI1640 culture medium (Sigma–Aldrich, St. Louis, MO, USA). The B-cell lymphoma/leukemia cell lines SU-DHL4, SU-DHL-6, SU-DHL10, TMD8 and Daudi were used as positive controls for CD20 expression. RRBL1[9–11] and WILL2[26] are cell lines established from B-cell lymphoma patients showing CD20-negative phenotypic changes after repeated chemotherapy with rituximab.

**Confirmation of CD20 protein expression with immunohistochemistry positive and flow cytometry analyses.** For IHC analysis, CD20 protein expression was confirmed using mouse anti-CD20 antibody L26 (Dako, Carpinteria, CA, USA). A pan-B-cell marker CD79a expression for the detection of B-cell was confirmed by anti-CD79a antibody (Dako). FCM analysis was performed with a BD FACSAria III cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA). For FCM, CD20 expression was confirmed with mouse anti-CD20 antibody B9E9 (a mouse monoclonal IgG2a antibody recognizing the B1 epitope [Beckman Coulter, Fullerton, CA, USA]) or

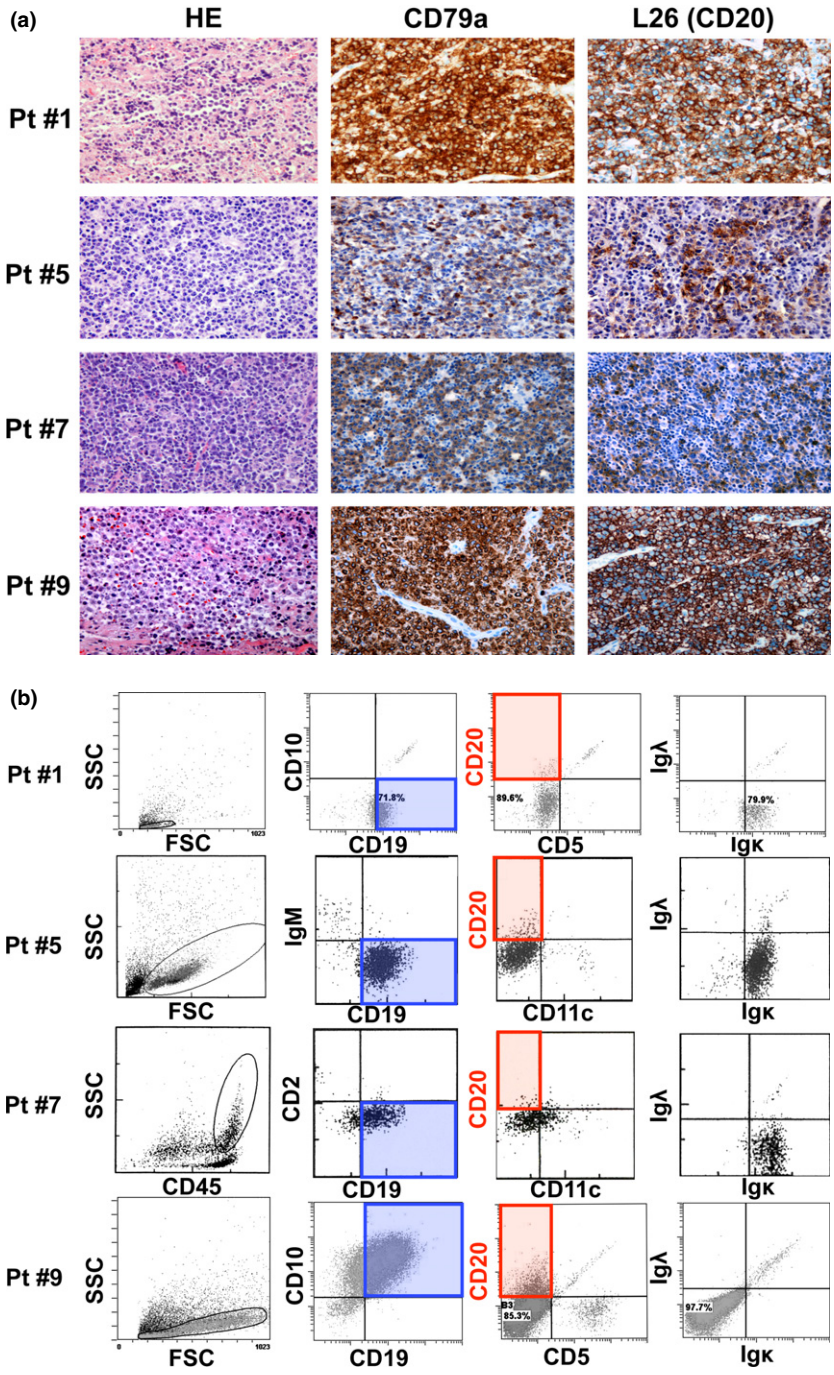


**Fig. 1.** Immunohistochemistry (IHC) and flow cytometry (FCM) analysis of *de novo* diffuse large B-cell lymphoma (DLBCL) patients with the CD20 IHC(+)/FCM(−) phenotype. Representative data for four patients are indicated. (a) IHC analysis using anti-CD79a and L26 (anti-CD20) antibody. All those patients were diagnosed as CD79a(+) and CD20(+) *de novo* DLBCL. (b) FCM analysis of patients showing the CD20 IHC(+)/FCM(−) phenotype. B-cell lymphoma cells were confirmed by gating of SSC, FSC or CD45 expression levels, as well as the CD19-positive phenotype. CD20 expression in those cells was significantly low with FCM analysis. FSC, forward scatter; HE, hematoxylin–eosin staining; Ig, immunoglobulin; L26, anti-CD20 antibody for IHC; Pt #, patient number; SSC; side scatter. Original magnifications (a); ×200 (Olympus BX51TF microscope, Olympus, Tokyo, Japan, and Nikon DS-Fi1 camera, Nikon, Tokyo, Japan).

B1 [Dako]). The percentages of negative and positive cells from FCM were determined after subtracting background from use of an isotypic control antibody (mouse IgG1 [Beckman Coulter]). B cell lymphoma cell population was basically confirmed by CD19 positivity in FCM analysis. FCM data of CD10, CD5, Igκ and Igλ were also referenced for lymphoma cell determination. If the percentage of CD20-positive cells in the tumor cell population was <12.5%, we considered those cells CD20 FCM negative. MFI of CD20 was measured with a BD FACSAria III cell sorter.

**DNA, RNA and protein extraction from lymphoma tissues.** Genomic DNA from tumor cells was extracted as described.[10]

**Immunoblotting.** Immunoblotting using whole-cell lysates of lymphoma cells was performed as described previously.[9,10,27]

***In vitro* CDC assay.** For the CDC assay, $1.0 \times 10^6$ cells were resuspended in 500 μL normal human serum and the same amount of complete medium with 10 μg/mL rituximab at 37°C for 30 min. Normal human serum was obtained from healthy volunteer donors. Dead cells were evaluated with DAPI and Annexin V-FITC staining. Briefly, cells placed in 96-well plates were stained with 2 μg/mL DAPI and 2 μg/mL Annexin V-FITC for 15 min at room temperature in the dark and evaluated with FCM (FACSCalibur or FACSAriaII [BD]).

Detailed information of analytical procedures is also indicated in the Data S1 and S2.

## Results

***De novo* diffuse large B-cell lymphoma patients with the CD20 IHC(+)/FCM(−) phenotype.** CD20 protein expression was confirmed with IHC using L26 antibody for all *de novo* DLBCL patients diagnosed in Nagoya University Hospital ($n$ = 106) (Table 1). If sufficient lymphoma materials were harvested at diagnosis, FCM analysis was also performed ($n$ = 37; 34.9%). Of those 37 cases, 8 (21.6%) were CD20-negative with FCM analysis, despite the CD20-positive phenotype with IHC. A CD20 IHC(−)/FCM(+) phenotype was not observed in this analysis. These results indicated that the CD20 IHC(+)/FCM(−) phenotype was not rare in *de novo* DLBCL patients.

Primary or cryopreserved lymphoma tissues showing the CD20 IHC(+)/FCM(−) phenotype obtained in Nagoya University Hospital ($n$ = 8) and the affiliated hospitals ($n$ = 4) were used for further analyses (Table 2). Representatives of this phenotype are shown in Figure 1(a) (IHC) and 1(b) (FCM). For IHC analysis, B cells were confirmed with anti-CD79a antibody, which recognizes a B-cell receptor component. CD20 protein expression was also confirmed in CD79a-positive B cells (Fig. 1a). For FCM analysis, lymphoma cells were gated by side scatter and forward scatter or the CD45 expression level, and CD19-positive B-cell lymphoma populations were confirmed (Fig. 1b). However, CD20 expression was not confirmed in these cell populations. B-cell light chain restriction was also confirmed with FCM, and 4 out of 12 cases (33.3%) expressed neither kappa-light chains nor lambda-light chains (Fig. 1b and Table 2). Interestingly, seven out of eight patients who expressed either the kappa or lambda chain expressed the kappa chain (87.5% of light chain-expressing patients). This percentage in CD20 IHC(+)/FCM(−) patients was higher tendency than that in CD20 IHC(+)/FCM(+) patients (nine out of 20 patients in Table 1 [45.0%]).

**Lower expression of *CD20* mRNA and protein in CD20 IHC(+)/FCM(−) B-cell lymphoma cells.** Total RNA was prepared from CD20 IHC(+)/FCM(−) lymphoma cells for RT-PCR analysis. Semi-quantitative RT-PCR indicated that *CD20* (*MS4A1*) mRNA expression was generally lower in CD20 IHC(+)/FCM(−) cells than that in CD20 positive control cells (Fig. 2a). Quantitative RT-PCR was also performed (Fig. 2b). Note that CD20 IHC(−)/FCM(−) cells were harvested from patients who showed a CD20-negative phenotypic change after repeated rituximab treatment and who showed clinical resistance to rituximab.[9,10] *CD20* mRNA expression was significantly lower

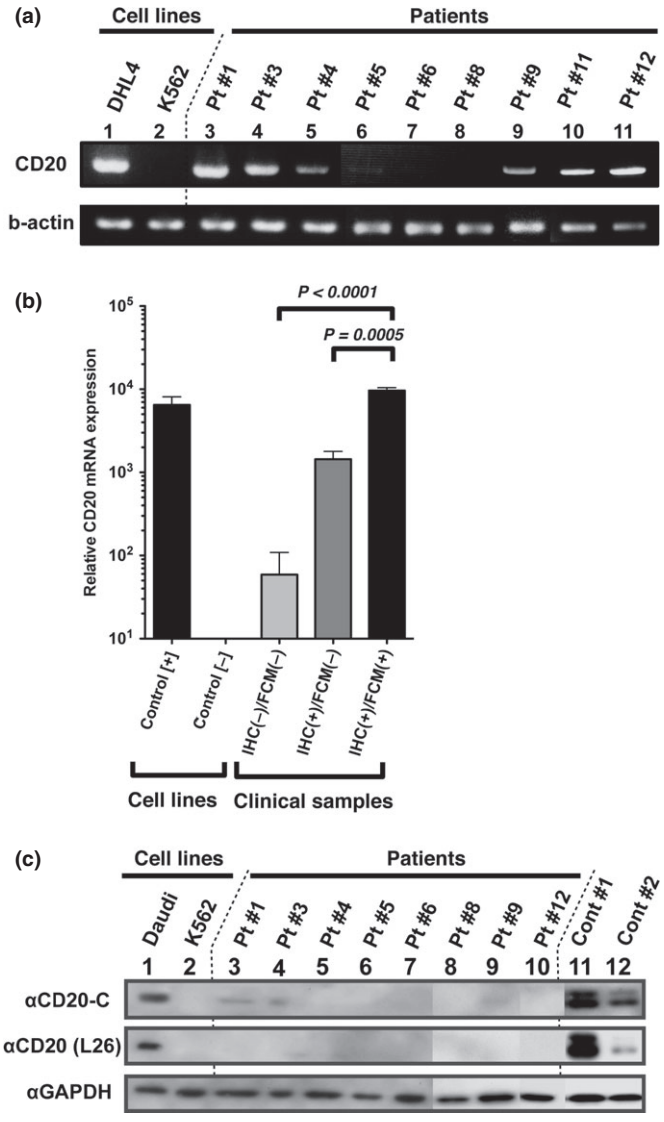


**Fig. 2.** Confirmation of *CD20* mRNA and protein expression with (a) semi-quantitative RT-PCR, (b) quantitative RT-PCR and (c) immunoblotting. Total mRNA and protein lysates were obtained from primary lymphoma samples for RT-PCR and immunoblotting. (a) The coding sequence of *MS4A1* (*CD20*) mRNA was amplified using semi-quantitative RT-PCR. *Beta actin* mRNA was amplified as an internal control. (b) Quantitative RT-PCR for *MS4A1* gene expression was performed. As an internal control, *GAPDH* expression was analyzed, and all data were normalized to its expression. (c) Immunoblotting was performed to confirm the CD20 protein expression. CD20-C recognizes the C-terminal region of the CD20 protein. The L26 antibody, which recognizes intracellular domains of the CD20 protein, was also used in this assay in addition to immunohistochemistry (IHC) analysis. Proteins from the Daudi and K562 cell lines were used as positive and negative controls, respectively. Cont #1 and #2 were derived from diffuse large B-cell lymphoma (DLBCL) clinical samples showing the CD20 IHC(+)/FCM(+) phenotype.

in CD20 IHC(+)/FCM(−) cells than in IHC(+)/FCM(+) cells ($P$ = 0.0005) and tended to be higher than in IHC(−)/FCM(−) cells (not significant).

Immunoblotting analysis using two anti-CD20 antibodies that recognize different domains of the CD20 protein indicated that CD20 expression was generally lower in lymphoma samples showing the CD20 IHC(+)/FCM(−) phenotype than in positive control samples from patients showing the CD20 IHC (+)/FCM(+) phenotype (Fig. 2c, lanes 3 to 10 *vs* lanes 11 and 12). Bands showing faint CD20 expression were confirmed with immunoblotting after a longer exposure (data not shown). These data suggest that lower *MS4A1* gene expression may contribute to the lower CD20 protein expression in CD20 IHC (+)/FCM(−) cells, as seen with immunoblotting and FCM analyses. These results also indicate that CD20 protein accumulation in the cytoplasm is not a likely explanation for the CD20 FCM(−) phenotype.

**Rituximab recognizes the CD20 cell surface antigen more readily than the B1 antibody with flow cytometry analysis.** To confirm the rituximab effectiveness on CD20 IHC(+)/FCM(−) cells, we first performed FCM analysis using fluorescent (Alexa 488)-labeled rituximab in addition to a conventional anti-CD20 antibody B1 (Dako) (Fig 3). We used primary B-cell lymphoma cells and cell lines showing the following phenotypes: CD20 IHC(+)/FCM(+) (primary; $n$ = 10, cell lines; $n$ = 3), IHC(+)/FCM(−) (primary; $n$ = 5) and IHC(−)/FCM (−) after using rituximab (cell lines; $n$ = 2). When using the B1 antibody (Fig. 3a), the MFI of CD20 IHC(+)/FCM(−) cells was significantly lower than that of IHC(+)/FCM(+) cells ($P$ = 0.03), consistent with the result of FCM analysis using the B9E9 antibody that recognized the B1 epitope of the CD20 protein (Fig. 1b). Using the same cell samples, FCM analysis using Alexa 488-labeled rituximab was also performed (Fig. 3b). The MFI of CD20 IHC(+)/FCM(−) cells showed a much lower tendency than that of IHC(+)/FCM(+) cells, but the difference was not significant ($P$ = 0.21). Rituximab, as well as B1, did not detect CD20 expression in the IHC(−) /FCM(−) B-cell lines, RRBL1 and WILL2. These data suggest that CD20 protein is faintly expressed on the surface of CD20 IHC(+)/FCM(−) cells and that rituximab can detect CD20 on the cell surface more effectively than the B1 (B9E9) antibody, even when the expression is very faint.

**CDC activity induced by rituximab is partially effective on CD20 IHC(+)/FCM(−) lymphoma cells.** We next performed a rituximab-induced *in vitro* CDC assay using the same primary lymphoma cells and cell lines as in Figure 3. Cells were cultured with or without rituximab for 30 min, and the dead cells were calculated by counting Annexin V- and PI- (or DAPI-) positive cells. Representative data are depicted in Figure 4(a). Almost 100% of CD20-positive control SU-DHL4 cells were killed by rituximab-induced CDC activity, but CD20-negative K562 and WILL2 cells were not killed under the same conditions. For the CD20 IHC(+)/FCM(−) cells, partial cell death was observed (Fig. 4a, #8). Because normal T cells and/or stromal cells were contaminating cell types in this assay when using primary lymphoma cells from lymphoma tissues, normalization to the B-cell population percentage estimated by determining the CD19-positive cell population was required (data not shown). This normalization for the percent of rituximab-induced cell death was performed for all data obtained from primary lymphoma samples. The relationship between CD20 MFI and the percent of cell death by rituximab-induced CDC activity is indicated in Figure 4(b) (MFI; B1) and 4(c) (MFI; rituximab). From these data, a positive correlation was confirmed between the CD20 MFI level and the rituximab effectiveness, as reported previously.[23,24] Importantly, rituximab was partially effective on CD20 IHC(+)/FCM(−) cells *in vitro* (cell death%; range 47–81%) compared to IHC(+) /FCM(+) cells (68–100%) (Fig. 4b,c). Significantly lower efficacy of rituximab in the CDC assay in IHC(+)/FCM(−) cells compared with IHC(+)/FCM(+) cells was confirmed ($P$ < 0.05) (Fig. 4d). CDC activity was not observed in CD20 IHC(−)/FCM(−) RRBL1 and WILL2 cells (black diamonds in Fig. 4b,c). These data suggest that rituximab-induced cytotoxicity can be observed with this CDC assay if the CD20 expression is confirmed with rituximab FCM analysis.

**No significant difference was observed in the overall survival rate between CD20 IHC(+)/FCM(+) and IHC(+)/FCM(−) patients.** The OS rate was analyzed using Kaplan–Meier analysis (Fig. 5). All DLBCL patients analyzed ($n$ = 106) were treated

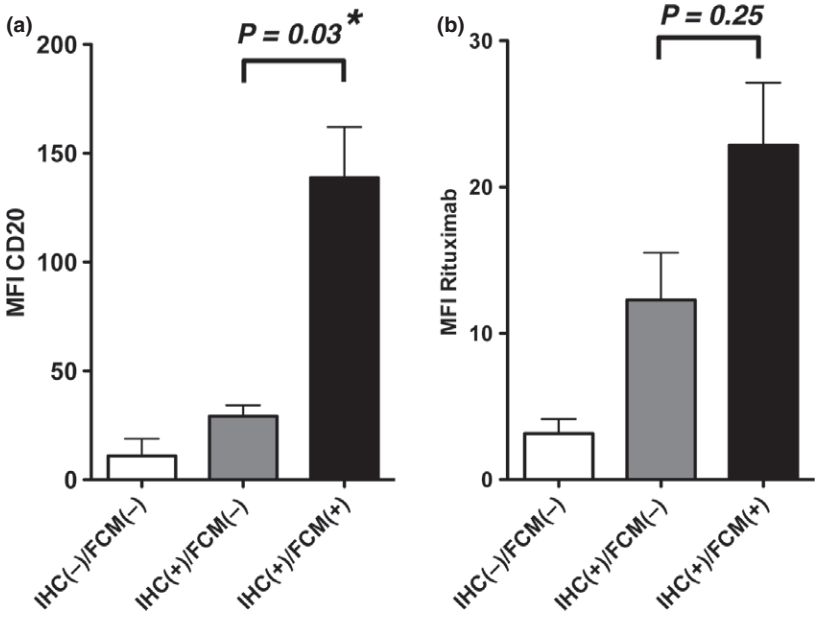


**Fig. 3.** Flow cytometry (FCM) analyses using anti-CD20 B1 antibody and fluorescent-labeled rituximab. (a) FCM analysis using anti-CD20 B1 antibody was performed, and the MFIs of lymphoma cells were measured. RRBL1 and WILL2 cells were utilized as representative CD20 IHC(−)/FCM(−) samples. The $P$-value is shown, and the asterisk indicates a statistically significant difference. (b) The MFI value using Alexa 488-labeled rituximab was also analyzed in the same lymphoma samples as (a).

with rituximab and CHOP-based combination chemotherapy at Nagoya University Hospital. The OS and PFS of all these patients at 3 years were 77% and 65.2%, respectively (Fig. 5a, b). The OS and the PFS of each group classified by the IPI[28] are indicated in Figure 5(c,d). Patients with the IHC(+)/FCM(−) phenotype tended to show a lower survival rate than IHC(+)/FCM(+) patients, but no significant difference was found between these two groups ($P = 0.664$) (Fig. 5e).

**Mouse xenograft model of human diffuse large B-cell lymphoma with the CD20 IHC(+)/FCM(−) phenotype.** A mouse xenograft model of human DLBCL was obtained by injecting primary DLBCL cells from the bone marrow of patient #8 (Table 2) into the intra-peritoneal space of NOD/SCID mice (see Fig. S1).

## Discussion

In this report, we showed that *de novo* DLBCL patients with the CD20 IHC(+)/FCM(−) phenotype are not rare, with a frequency of occurrence of 21.6% in patients analyzed with both IHC and FCM at diagnosis. Previous reports indicate the same phenomenon. Johnson *et al.* report that 16% of *de novo* DLBCL patients analyzed with both IHC and FCM (B9E9 antibody) showed reduced CD20 expression in FCM analyses despite a positive result with IHC analysis.[25] Miyoshi *et al.* describe the relationship between the CD20 IHC-positive score and the FCM-positive (using B-Ly1 mouse monoclonal IgG1 antibody) rate in patients with *de novo* and relapsed DLBCL and follicular lymphoma.[21] They also show that lower expression of CD20 with FCM is observed even with a higher IHC positive score. These reports and our data indicate that a discrepancy in protein expression analysis between IHC and FCM is a common phenomenon in DLBCL in the clinical setting.

Genetic mutations of *MS4A1* have been speculated to be a molecular mechanism of the CD20 FCM-negative phenotype. Genetic mutations may lead to protein conformational changes in the CD20 protein. In particular, amino acid substitution in the large outer loop of CD20 may directly affect the effectiveness of antibody recognition,[14,15,29,30] and mutations in the intracellular domain may lead to aberrant protein localization.[14] We performed mutation analysis for the CD20 coding sequences (exons 3 to 8), and no mutations were found in patients with the CD20 IHC(+)/FCM(−) phenotype. Previous reports also indicate no significant missense or nonsense mutations in

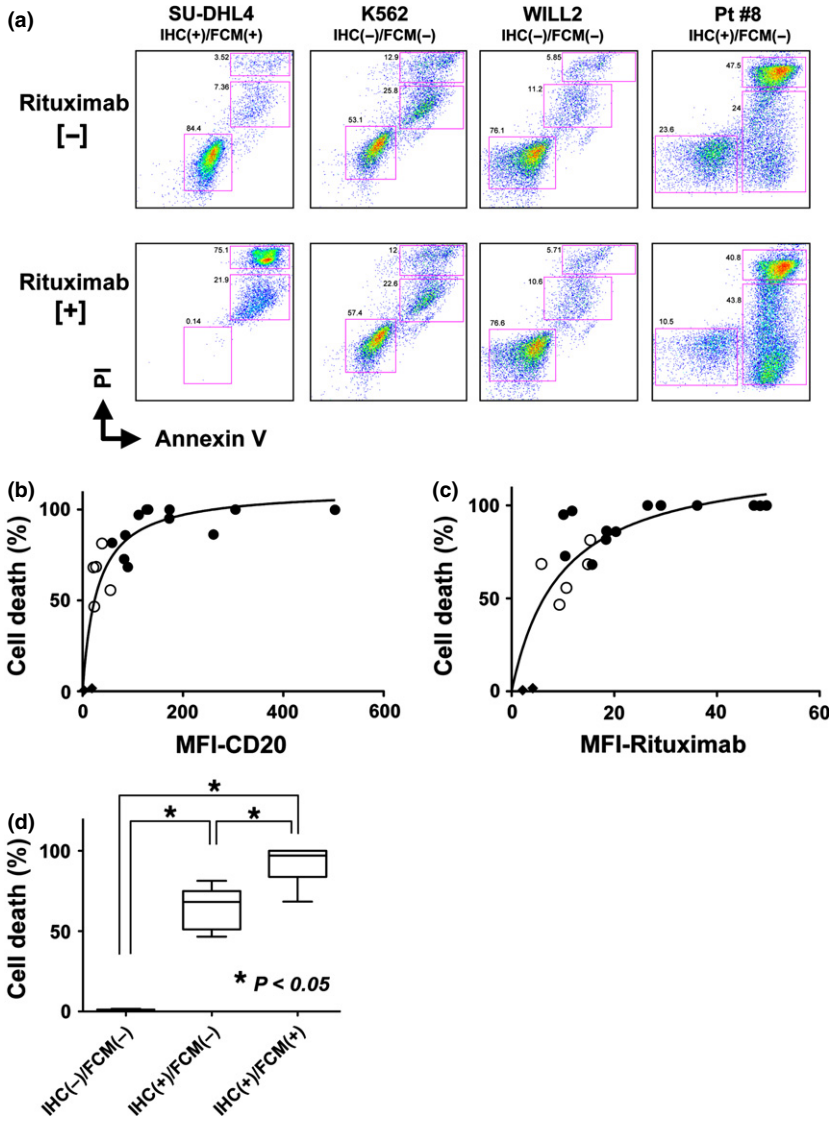


**Fig. 4.** *In vitro* CDC activity induced by rituximab. (a) Annexin V-PI staining was performed with/without rituximab and human serum treatment *in vitro*. In this assay, living, pro-apoptotic, or dead cell populations were separated in a 2-dimensional graph, and the percentage of each group was calculated. The SU-DHL4 cell line was a positive control. The K562 and WILL2 cell lines were negative controls. Representative primary lymphoma cells showing the IHC(+)/FCM(−) phenotype were obtained from patient #8 and utilized in this assay. (b) The relationship between the percent of cell death with rituximab-induced CDC activity and the CD20-B1-MFI value (performed in Fig. 3) was plotted in this graph. Primary lymphoma samples showing CD20 IHC(+)/FCM(+) (black circles) and IHC(+)/FCM(−) (white circles) were used. Each circle indicates one lymphoma sample from a corresponding patient. RRBL1 and WILL2 cells are indicated in black diamonds. (c) The same analysis using the rituximab-MFI values is shown. Nonlinear regression curve fitting is indicated as curved lines. (d) Cell death percentages were statistically compared using Turkey's multiple comparison test. Asterisks indicate significant differences.

*MS4A1*.[21,25] These data indicate that genetic mutations in *MS4A1* are not the explanation for the CD20 IHC(+)/FCM(−) phenotype in *de novo* DLBCL. Nakamaki *et al.*[16] report copy number loss of *MS4A1* located at 11q12 in a specific patient who showed the CD20-negative phenotype after treatment with rituximab. Conventional chromosomal analysis showed that 11q12 genetic loss was not detected in patients with the IHC(+)/FCM(−) phenotype (data not shown). It remains possible that copy number loss of *MS4A1* may, in part, be related to the lower *CD20* mRNA expression in some *de novo* DLBCL patients with the CD20 IHC(+)/FCM(−) phenotype.

Quantitative RT-PCR analyses using primary lymphoma cells indicated that *MS4A1* mRNA expression was significantly lower in CD20 IHC(+)/FCM(−) cells than in IHC(+)/FCM(+) cells (Fig. 2b). Lower *CD20* mRNA expression possibly meant that *CD20* mRNA and protein expression was not confirmed in several samples from CD20 IHC(+)/FCM(−) cells in semi-quantitative RT-PCR (Fig. 2a) and immunoblotting (Fig. 2c). Because pan-B and C-terminal antibodies were used to detect CD20 protein in this assay, the possibilities of internalization of the protein into the cytoplasm and truncation of the protein can be mostly excluded as reasons for this phenotype. We did not examine why *CD20* mRNA expression was repressed in those B cells, but possible explanations are as follows: (i) aberrant expression of transcription factors critical for *MS4A1* expression such as IRF4, Pu.1, Pip[11,31] and transforming growth factor-beta;[32] (ii) abnormal epigenetic modulation by histone acetylation, methylation and DNA methylation at the *MS4A1* promoter;[11] and (iii) deregulation of normal cell differentiation into mature B cells. Using FCM analysis, 4 out of 12 patents with the CD20 IHC(+)/FCM(−) phenotype showed no light chain restrictions (Fig. 1b and Table 2). This finding suggests that some aberrant genetic and/or epigenetic mechanisms that downmodulate the light chain expression on lymphoma cells might correlate with this phenomenon. Further molecular analyses are required to demonstrate those possibilities.

An important question is whether significantly lower protein expression results in discrepancy in the data of IHC and FCM analyses. One likely explanation for this phenomenon is that the sensitivity for detecting CD20 protein is much higher with IHC using L26 than that with FCM using B9E9 and B1. If the expression is high enough, both analyses will indicate positive results, and if the *CD20* mRNA level is almost 100 times lower than that in CD20 IHC(+)/FCM(+) cells, neither IHC nor FCM can detect CD20 protein expression, as seen in RRBL1 and WILL2 cells.[9,26] If the *CD20* mRNA expression level is almost 10 times lower than that in IHC(+)/FCM(+) cells, the anti-CD20 antibodies B9E9 and B1 in FCM may not sufficiently recognize the CD20 protein. Recent reports indicate that some newer generation antibodies such as ofatumumab,[2,33] GA101[34] and HuMab-7D8[24] show significantly higher cytotoxic activity than rituximab, even in the population of cells with lower CD20 protein expression. From these findings, using ofatumumab, GA101 and HuMab-7D8 may be a good strategy to overcome the partial rituximab resistance of CD20 IHC(+)/FCM(−) cells.

Interestingly, when using fluorescent-labeled rituximab in FCM, the difference in MFI between CD20 IHC(+)/FCM(−) and IHC(+)/FCM(+) cells was significantly smaller than that of FCM using the B1 antibody, indicating that the sensitivity of CD20 protein recognition by rituximab is much higher than

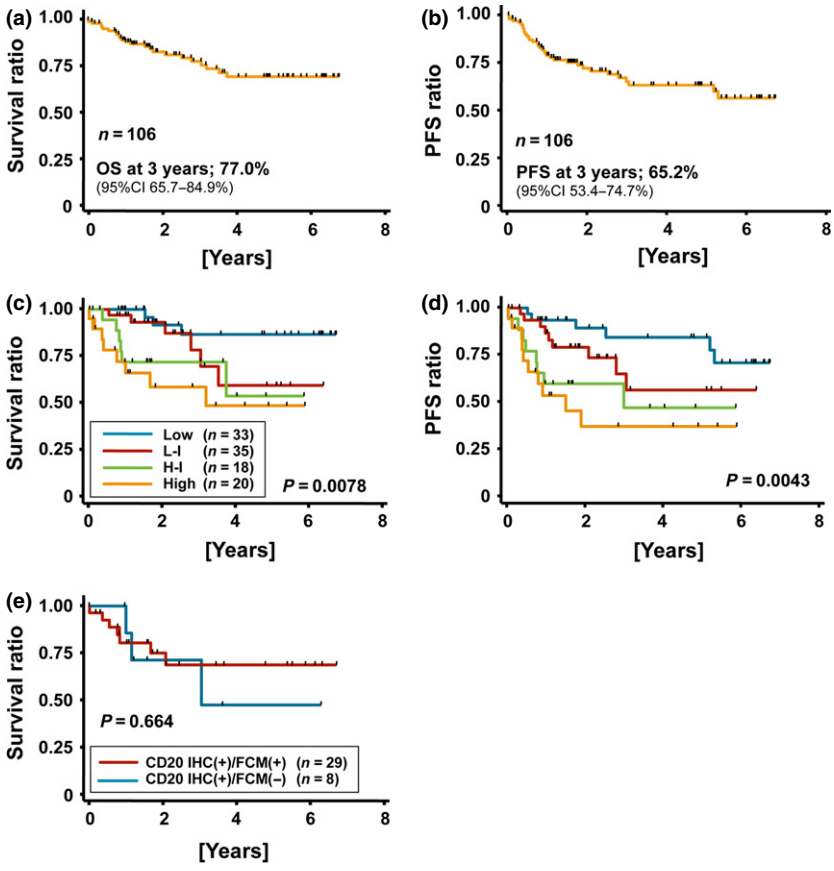


**Fig. 5.** Prognosis of diffuse large B-cell lymphoma (DLBCL) patients with the CD20 IHC(+)/FCM(−) phenotype. (a) overall survival (OS) and (b) progression free survival (PFS) of DLBCL patients diagnosed in Nagoya University Hospital ($n$ = 99). All patients were treated by combination chemotherapy with rituximab. These patients were classified by IPI, and the OS and PFS of each group are indicated in (c) and (d), respectively. (e) Comparison of OS of DLBCL patients who were diagnosed using both immunohistochemistry (IHC) and flow cytometry (FCM) ($n$ = 36).

that of the B1 and B9E9 antibodies. Because the partial efficacy of rituximab in inducing CDC activity was confirmed even in the CD20 IHC(+)/FCM(−) cells in the *in vitro* assay (Fig. 3), utilization of rituximab for patients with the IHC(+)/FCM(−) phenotype may be still recommended in the clinical setting. Furthermore, using fluorescent-labeled rituximab in FCM at diagnosis may be much more informative than using B1/B9E9 to predict the rituximab effectiveness *in vivo*.

Our analysis showed no significant difference in OS between patients with CD20 IHC(+)/FCM(+) and IHC(+)/FCM(−) phenotypes (Fig. 5), despite the significantly lower cytotoxic activity of rituximab on CD20 IHC(+)/FCM(−) cells compared to IHC(+)/FCM(+) cells (Fig. 4d). Considering the *in vitro* CDC analysis, combination strategies with conventional chemo-regimens such as CHOP may improve the poor responsiveness to rituximab therapy, and, furthermore, ADCC and the direct signal transduction resulting in apoptosis can be induced in addition to CDC activity *in vivo*. Considering our clinical and *in vitro* data, rituximab utilization combined with chemotherapy is still recommended even for patients showing the CD20 IHC(+)/FCM(−) phenotype.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1.** Mouse xenograft model of human DLBCL with the CD20 IHC(+)/FCM(−) phenotype.

**Data S1.** Methods.

**Data S2.** Results and legend.