CD20 is a differentiation related cell surface phosphoprotein that is expressed during early pre-B cell stages until plasma cell differentiation, and is a suitable molecular target for B-cell malignancies by monoclonal antibodies such as rituximab, ofatumumab, obinutuzumab and others. CD20 expression is confirmed in most B-cell malignancies; however, the protein expression level varies in each patient, even in de novo tumors, and down-modulation of CD20 expression after chemoimmunotherapy with rituximab, resulting in rituximab resistance, has been recognized in the clinical setting. Recent reports suggest that genetic and epigenetic mechanisms are correlated with aberrantly low CD20 expression in de novo tumors and relapsed/refractory disease after using rituximab. Furthermore, some targeting drugs, such as lenalidomide, bortezomib and ibrutinib, directly or indirectly affect CD20 protein expression. CD20-negative phenotypically-changed DLBCL after rituximab use tends to show an aggressive clinical course and poor outcome with resistance to not only rituximab, but also conventional salvage chemo-regimens. Understanding of the mechanisms of CD20-negative phenotype may contribute to establish strategies for overcoming chemo-refractory B-cell malignancies. In this review, recent progress of research on molecular and clinical features of CD20 protein and CD20-negative B-cell malignancies was reviewed. [J Clin Exp Hematop 56(2):89-99, 2016]

Keywords: CD20, MS4A1, rituximab, drug resistance, epigenetic drugs
antibodies. The CD20 homodimer and homotetramer exist as a protein complex with other proteins on the surface of B-cells, and contribute to signal transduction.\textsuperscript{12,16,17} Binding of anti-CD20 monoclonal antibodies with CD20 induces cell cycle arrest,\textsuperscript{18} differentiation block or B-cell activation\textsuperscript{19} through phosphorylation of CD20,\textsuperscript{20} lipid raft localization of CD20 on cell membrane\textsuperscript{21,22} and inducing intracellular calcium flux.\textsuperscript{23} The responses of monoclonal antibodies are different depending on the antibodies used\textsuperscript{22} and/or the disease condition of each patient.

CD20 (B1) was first identified as a B-cell specific differentiation antigen recognized by a monoclonal antibody (anti-B1).\textsuperscript{24} Anti-B1 antibody is utilized for flow cytometry (FCM) analysis to recognize the extracellular domain of CD20. Ishii et al. purified B-cell specific TB2-2B3 monoclonal antibody,\textsuperscript{25} which recognized the B-cell specific antigen L26. Anti-L26 antibody appeared to recognize the intracellular domain of CD20,\textsuperscript{26} and is now widely utilized for immunohistochemistry (IHC) analyses in the clinical setting.

\textbf{CD20 EXPRESSION IN B-CELL MALIGNANCIES}

CD20 protein expression is detected in most B-cell malignancies.\textsuperscript{15} However, the expression level is varies in each patient, even in those with the same diagnosis.\textsuperscript{27-34} Especially in chronic lymphocytic leukemia (CLL), the mean expression level of CD20 in FCM is significantly lower than that in DLBCL, FL and other B-cell malignancies (Fig. 2).\textsuperscript{27} Miyoshi et al.\textsuperscript{28} reported that CD20 protein expression confirmed by IHC and FCM in B-cell malignancies shows wide variation among patients. \textit{De novo} DLBCL with CD20-negative phenotype in IHC is reported in limited cases (less than 2%), and the prognosis appeared to be significantly poor.\textsuperscript{33-35} \textit{De novo} DLBCL showing the specific phenotype of CD20 IHC-positive, but FCM-dim-negative (IHC+/FCM-), was also reported by several independent groups.\textsuperscript{28-30,33} Tokunaga et al. concluded that an approximately 10-times lower expression of CD20 mRNA compared to CD20 IHC+/FCM+ control cells is likely the main molecular background of this phenotype.\textsuperscript{29} Prognosis of this DLBCL phenotype by chemo-immunotherapy is still controversial.\textsuperscript{29,30,33}

Aberrant down-modulation of CD20 protein expression in B-cell malignancies after rituximab use\textsuperscript{10,28,30,36-44} and other
targeting drugs\textsuperscript{45-47} has been observed in the clinical setting and also \textit{in vitro} (Table 1). This will be discussed hereinafter.

**MOLECULAR MECHANISMS OF RITUXIMAB RESISTANCE IN B-CELL MALIGNANCIES**

Significant numbers of patients with B-cell malignancies show RD/PD even after chemotherapy with rituximab, and many of those cannot be cured. Acquisition of rituximab resistance may be one of the critical reasons for RD/PD. Putative mechanisms of action and resistance to rituximab are depicted in Fig. 3. CD20 molecules localized on the cell membrane are recruited onto the lipid raft just after rituximab binding,\textsuperscript{21,22,48} followed by signal transduction into the cytoplasm and calcium flux from the intracellular calcium pool.\textsuperscript{23}

After binding of rituximab, the complement complex is recruited to rituximab to form a membrane attack complex, and complement dependent cytotoxicity (CDC) occurs.\textsuperscript{1,49,50} Furthermore, effector cells, such as natural killer cells and macrophages, bind with the Fc portion of rituximab through Fc receptors, and antibody dependent cell mediated cytotoxicity (ADCC) occurs.\textsuperscript{1,49,50}

Two major mechanisms of rituximab resistance are speculated as follows: 1) abnormalities of rituximab binding with CD20, and 2) abnormality of mechanisms after rituximab binding with CD20. For examples of 1), genetic mutations in the coding region of the \textit{MS4A1} gene,\textsuperscript{28,40,51} aberrant expression of splicing variants of \textit{CD20} mRNA,\textsuperscript{13} likely resulting in conformational change of the rituximab binding epitope, and alteration of the localization of CD20 on the membrane, are reported. Down-modulation of \textit{MS4A1} gene

### Table 1. Modulation of CD20 protein expression in B-cell malignancies

<table>
<thead>
<tr>
<th>CD20 expression modulating factors</th>
<th>Putative mechanisms of CD20 expression modulation</th>
<th>References</th>
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<td><strong>Type I antibodies</strong></td>
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| Rituximab                         | • Down-regulation of \textit{MS4A1} gene expression by epigenetic mechanisms  
                                 | • Internalization of CD20 molecule  
                                 | • Selection of CD20-negative clones  
| **Molecular targeting therapeutics** |                                                  |            |
| Ibrutinib                         | • Inhibition of NF\(\kappa\)B related factors that is critical for CD20 expression | Skarzynski 2015 |
| Lenalidomide                      | • Internalization of CD20 molecule               | Lapalombella 2008 |
| Bortezomib                        | • Protein degradation by lysosomal/autophagic mechanisms | Bli 2010 |
| **Genetic mutations**             |                                                  |            |
| \textit{MS4A1} gene mutation / deletion | • C-terminal deletion that may be critical for large loop conformation  
                                 | • Exon 5 mutation that encodes large loop  
                                 | • Deletion of 11q12  
| \textit{NOTCH1} mutation          |                                                  |            |
| **Others**                        |                                                  |            |
| Smad2/3 expression                | • Recruitment of Smad complex by TGF\(\beta\) signal induce \textit{MS4A1} gene repression | Kawabata 2012 |
| Splicing variant                  | • Expression of the short form of CD20 that lacks exons 3 to 7. | Henry 2010 |
| **HDAC inhibitors**               |                                                  |            |
| Trichostatin                      | • De-repression of \textit{MS4A1} gene promoter by inhibiting HDACs recruited by transcription factors  
                                 | • Stimulation of expression of \textit{MS4A1} targeted transcription factors* | Tomita 2007, Sugimoto 2009, Granata 2013, Damm 2015 |
| Valproic acid                     |                                                  |            |
| Rhomidepsine                      |                                                  |            |
| **Demethylating agents (DNMT inhibitors)** |                                                  |            |
| Azacitidine                       | • De-repression of \textit{MS4A1} gene through CpG de-methylation induced by DNMTs**  
                                 | • Stimulation of expression of \textit{MS4A1} targeted transcription factors* | Hiraga 2009, Sugimoto 2009, Granata 2013 |
| Decitabine                        |                                                  |            |
| **Other drugs**                   |                                                  |            |
| Farnesyltransferase inhibitors (L-744, 832) | • Recruitment of Pu.1/Oct2 transcription factors to \textit{MS4A1} gene promoter | Winiarska 2012 |
| CpG oligodeoxynucleotides         | • Stimulation of \textit{MS4A1} gene expression by other than induction of Pu.1 | Markaki 2009 |
| TGF\(\beta\) inhibitor (LY364947) | • Inhibition of Smad2/3 recruitment to \textit{MS4A1} gene promoter | Kawabata 2012 |

\textit{MS4A1}, membrane spanning 4-domains A1; HDAC, histone deacetylase; DNMT, DNA methyltransferase

* Specific transcription factors have not been identified.

** Specific position of CpG methylation that affect \textit{MS4A1} gene expression has not been confirmed.
expression through epigenetic and/or genetic mechanisms resulting in a decrease of CD20 protein expression, especially after using rituximab, has been reported from several groups.\textsuperscript{10,36,38,41,52} Furthermore, internalization or degradation of CD20 protein after treatment with several targeting drugs,\textsuperscript{46,47,53} and shaving of the CD20-rituximab complex from the tumor cell surface by monocytes\textsuperscript{54,55} have been reported. It is also speculated that some specific genetic mutations in B-cell malignancies are correlated with down-regulation of MS4A1 gene expression.\textsuperscript{56} For examples of 2), abnormalities in lipid raft localization of CD20, signal transduction, calcium flux and apoptosis are speculated as tumor cell dependent issues (\textsuperscript{23} and author’s unpublished data). Insufficient CDC activity by increasing expression of complement regulatory proteins, such as CD55/CD59 and others, is also speculated.\textsuperscript{57-61} Less effective ADCC activity due to Fc receptor polymorphisms is reported as a host condition.\textsuperscript{62,63} As many genetic mutations have been recently reported in B-cell malignancies,\textsuperscript{64-67} it is of interest whether some of them may affect rituximab sensitivity, especially in patients showing refractory diseases such as double hit lymphoma or histologically transformed FL.

GENETIC ABNORMALITIES OF THE MS4A1 GENE IN B-CELL MALIGNANCIES

In several patients with DLBCL, genetic mutations in the coding regions of the MS4A1 gene were identified. Terui et al.\textsuperscript{11} reported that 11 out of 50 (22%) previously untreated or RD/ PD non-Hodgkin’s B-cell malignancies carried genetic mutations. Interestingly, a C-terminal deletion mutation was confirmed in 4 cases (8%) with RD/ PD, and CD20 expression was significantly lower than in those with no mutations, suggesting that the C-terminal deletion may contribute to rituximab resistance through lower binding affinity with rituximab. Mishima et al.\textsuperscript{68} demonstrated that CD20 C-terminal deletion correlated with a decrease in large extracellular loop presentation on the cell membrane, resulting in rituximab resistance. Nakamaki et al.\textsuperscript{40} reported a patient who exhibited CD20-negative phenotypic change after chemotherapy with rituximab because of a homozygous deletion of the MS4A1 gene defined at the relapsed period. On the other hand, it is also reported that genetic mutations in the extracellular loop of CD20 are rare. Johnson et al.\textsuperscript{32} reported that genetic mutations on the rituximab epitope were confirmed in only 1 out of 264 (0.4%) and 1 out of 15 (6%) DLBCL patients at diagnosis and relapsed period, respectively. They concluded that CD20 mutations cannot represent a significant cause of R-CHOP resistance. Sar et al.\textsuperscript{51} reported that 11 DLBCL patients with poor outcome did not have any mutations in the MS4A1 gene coding exons.

DOWN-MODULATION OF CD20 PROTEIN EXPRESSION AFTER USING RITUXIMAB

Reports about phenotypic change of B-cell lymphoma causes...
into CD20-negative DLBCL after rituximab have been evaluated (Fig. 4A). Hiraga et al.10 reported that 36 out of 124 DLBCL patients showed RD/PD, and 5 out of 19 (26%) RD/PD patients receiving re-biopsy showed CD20-negative in IHC phenotypic change. Johnson et al.32 also reported that 66 out of 277 cases showed RD/PD and 3 out of 18 (16.7%) re-biopsied RD/PD patients showed CD20-negative phenotypic change. In these cases, relatively poor prognosis is observed. Previous limited reports and our experiments with more than 10 patients (unpublished data) indicated that B-cell lymphoma with CD20-negative phenotypic change diagnosed as DLBCL at RD/PD period showed very rapid progression with chemo-resistance, and most of the patients died of disease progression within 1 year after diagnosis of CD20-negative change.10,39,42,69 The duration of CD20-negative change after the last rituximab administration was from 1 to 81 months, and the administration times were from 4 to 14. In most of those patients, extranodal infiltration of CD20-negative cells, such as in bone marrow, central nervous system, liver, and skin, was observed. Furthermore, CD20-negative tumor cells were observed in peripheral blood, the so-called “leukemic stage”, at which their terminal stage was marked with serum lactate dehydrogenase (LDH) elevation (Fig. 4B). Laboratory data indicated that those tumors exhibited the CD20-negative phenotype by both IHC using L26 antibody and FCM using anti-B1 antibody, but were mainly the terminal deoxynucleotidyl transferase (TdT)-negative phenotype.10,39 CD20-negative transformed DLBCL may show an acute lymphoblastic leukemia (ALL)-like phenotype10 and clinical course; however, this phenotype should be distinguished with ALL based on differentiation stage. These clinical findings resemble aggressive refractory diseases, such as transformed-FL and double hit or Myc/Bcl2 double-protein-expression lymphoma,70-73 thus, it is of great interest whether this phenotype is correlated with specific additional genetic abnormalities. Further patient study and molecular analyses are required.

MOLECULAR BACKGROUNDS OF CD20-NEGATIVE PHENOTYPIC CHANGE AFTER RITUXIMAB

To explain the phenomenon of CD20 expression down-modulation after rituximab use, the following mechanisms were confirmed using patient cells and/or cell lines; down-regulation of MS4A1 (CD20) gene expression,10,52 internalization of the CD20-rituximab complex into the cytoplasm,74 and shaving of the CD20-extracellular domain-rituximab complex from tumor cells by monocytes54 were reported (Table 1). To date, 3 cell lines from CD20-negative transformed patients after rituximab use have been established (RRBL1,38 WILL239 and SD0770), and molecular mechanisms have been analyzed. RRBL1 cells are the cell line established from peripheral blood CD20-negative tumor cells of a patient.
CD20(+)

sis using anti-CD20 antibody (B2E9) was performed for B-cell lymphoma and used for molecular analysis. Flow cytometry (FCM) analysis of peripheral blood tumor cells from the patient indicated in Fig. 4B, showed a negative phenotypic change. RRBL1 cells were established from samples and CD20 protein and mRNA expression was confirmed by semi-quantitative real-time polymerase chain reaction (RT-PCR). Raji and NALM1 cells were used for positive and negative controls. CD20 mRNA expression was confirmed by quantitative RT-PCR using RRBL1 cells (also WILL2 cells) than in positive control patient cells (Fig. 5C & 5D), it was not zero. In vitro assays demonstrated that the CDC activity of rituximab was dependent on CD20 expression level, and RRBL1 and WILL2 cells were completely resistant (Fig. 6). These data suggested that aberrant transcription down-regulation may be one of molecular mechanisms of CD20-negative phenotypic change, resulting in rituximab resistance.

**STIMULATION OF CD20 EXPRESSION BY MOLECULAR TARGETING DRUGS**

Some epigenetic drugs, such as DNA methyltransferase (DNMT) inhibitors [5-azacytidine (5-Aza)] and 5-azadeoxycytidine (5-Aza-dC), and histone deacetylase (HDAC) inhibitors (trichostatin A (TSA), valproic acid and romidepsin) were evaluated in vitro for expected activation of MS4A1 gene expression through chromatin remodeling (Table 1).

In vitro analysis using RRBL1 cells, CD20 mRNA and protein expression were moderately stimulated by TSA and 5-Aza-dC, and the efficiency was enhanced by combination of these two drugs (Fig. 7A). Molecular analyses indicated that DNMT1 depletion occurred after administration of 5-Aza-dC as previously reported, followed by up-regulation of CD20 mRNA and protein expression (Fig. 7B). As there are no significant CpG islands in the MS4A1 gene promoter upstream (~ 5,000 bp) from the transcription start site, DNA demethylation by DNMT inhibitors on the MS4A1 gene may not be the reason for MS4A1 gene up-regulation. It was confirmed that the HDAC1-Sin3 co-repressor complex that was recruited by transcription factors dissociated from the MS4A1 gene promoter in the presence of 5-Aza-dC and TSA, followed by histone acetylation and transcription activation (Fig. 8). Recruitment of Pu.1 and IRF4, transcription factors that bind with MS4A1 gene promoters, was stable on the promoter site in both the presence and absence of these drugs. Shimizu et al. reported that HDAC inhibitors valproic acid and romidepsin can moderately stimulate MS4A1 gene expression by recruiting Sp1 to the promoter, resulting in hyperacetylation of histones to activate transcription. However, the induction efficacy of MS4A1 gene expression by the HDAC inhibitor was different in each cell line. Mankai et al. reported that CpG oligodeoxynucleotide stimulates CD20 mRNA and/or protein expression in a Pu.1 expression-independent manner. Winiarska et al. reported that farnesyltransferase inhibitors, L-744 and -832,
upregulate CD20 protein expression through Pu.1/Oct2 recruitment to the MS4A1 promoter and augment rituximab induced cytotoxicity.80

There are few reports using epigenetic drugs in the clinical setting for the purpose of stimulation of CD20 protein expression: for patients with CD20-negative FL,43 CD20-negative ALL75 and CD20-positive DLBCL.77 In these reports, CD20 protein expression level was moderately upregulated in each patient, but the efficiency varied among patients. Stimulation of CD20 expression by epigenetic drugs may improve CDC/ADCC activity by rituximab in vivo, however, the significance to clinical outcome remains to be confirmed.

As previously indicated, up-regulation of CD20 expression by HDACi and DNMTi, in vitro and in vivo, evokes epigenetic mechanisms in CD20-negative phenotypic change; however, there is little direct evidence about the contribution of epigenetic abnormalities in this phenotype. Another possibility is that genetic abnormalities in genes encoding epigenetic-related factors, such as TET2, IDH1/2, EZH2, and KMT2D (MLL2), which are sometimes mutated in myeloid and lymphoid malignancies, or in genes encoding transcription factors/co-regulators, which are critical for MS4A1 expression, may contribute to CD20 expression down-modulation. Further comprehensive analyses are required.

OTHER MECHANISMS AFFECTING CD20 EXPRESSION (Fig. 8, Table 1)

As many recurrent genetic mutations in B-cell malignancies are reported,64,66,67,81 whether CD20 down-modulation is correlated with specific gene abnormalities is of great interest. As described previously, CD20-negative transformed DLBCL tends to show aggressive features with chemo-resistance. These findings may suggest that some additional genetic mutations induce CD20 down-modulation and aggressiveness. To date, there are few reports suggesting this relationship. Pozzo et al.56 reported that NOTCH1 C-terminal (NICD; NOTCH1 intracellular domain) mutation82 in CLL was significantly correlated with lower expression of CD20 protein, and the CDC activity by rituximab and ofatumumab was significantly lower in NICD-mutation-positive CLL than in wild-type CLL. Mutated-NICD forms a protein complex with transcription factors RBPJ and HDAC1/2 on the MS4A1 gene promoter, and CD20 protein expression was repressed in NICD-mutated CLL patient.

![Fig. 7.](image)

**Fig. 7.** Epigenetic regulation of CD20 mRNA and protein expression. (7A) RRBL1 cells were treated with 5-aza-deoxycytidine (5-Aza-dC), a DNA methyltransferase (DNMT) inhibitor, and trichostatin A (TSA), a histone deacetylase inhibitor. CD20 protein expression was confirmed by flow cytometry. (7B) RRBL1 cells were treated with 5-Aza-dC, and CD20 mRNA and protein expression were confirmed by Western blotting and semi-quantitative real-time polymerase chain reaction. Note that CD20 expression was stimulated temporally after depletion of DNMT1 by DNMT inhibitor, and then decreased in a time-dependent manner.

![Fig. 8.](image)

**Fig. 8.** Mechanisms of CD20 mRNA down-regulation through transcription factors and transcription co-repressors (Co-R). Several transcription factors, such as IRF4, Pu.1 and Oct2, are recruited to the MS4A1 gene promoter and may contribute to transcription repression in the presence of Co-R.72,80,93 In few situations, Smad2/383 and RBPJ-mutated-NOTCH1 interact with the MS4A1 promoter, and contribute to transcription repression by recruiting Co-R. This repression can be upregulated by epigenetic drugs (DNA methyltransferase and histone deacetylase inhibitor inhibitors)10,38,52,75-77 and other drugs,80 probably through direct inhibition of Co-R that is recruited to the MS4A1 gene promoter and/or by indirect effects that modulate the expression of genes that are critical for MS4A1 gene expression.
cells. Kawabata et al. reported that activation of the TGFβ signaling pathway was negatively correlated with CD20 expression in B-cell non-Hodgkin lymphoma. They demonstrated that in Ramos cells, Smad2/3 were recruited to the MS4A1 promoter in the presence of TGFβ to repress transcription, and MS4A1 gene expression was stimulated by the TGFβ inhibitor, LY364947.

Several reports demonstrated that CD20 expression can be modulated by molecular targeting drugs. Beers et al. reported that CD20 on B-cell malignancies internalized after binding with type I anti-CD20 monoclonal antibodies (rituximab-like), resulting in reduced macrophage recruitment and degradation of CD20/antibody complexes. This phenomenon cannot be observed when using type II antibodies (tosilomomab-like), and was highly observed in CLL and mantle cell lymphoma compared with in FL or DLBCL.

CD20 protein internalization was also observed when lenalidomide, one of the celebro-interacting immunomodulatory drugs, was used on primary CLL cells without influencing MS4A1 transcription. Bil et al. reported that bortezomib induced CD20 protein degradation by lysozyme/autophagic mechanisms rather than the ubiquitin proteasome pathway, leading to reduced CDC activity by rituximab. As the effectiveness of lenalidomide and bortezomib, especially for non-germinal center B-cell type (activated B-cell type) DLBCL, was recently reported, CD20 protein expression level and rituximab sensitivity in rituximab-combination therapy should be evaluated in the future. Skarzynski et al. reported that ibrutinib, a Bruton tyrosine kinase (Btk) inhibitor that leads to NFκB pathway down-regulation, decreased CD20 protein expression on CLL cells in the clinical setting. They speculated that the NFκB consensus sequence was in the MS4A1 gene promoter, thus, inhibition of the NFκB signal pathway may contribute to the down-regulation of CD20 mRNA expression.

CONCLUSION

Although CD20 expression is required for the efficacy of anti-CD20 monoclonal antibody therapeutics, it can be easily modulated by disease condition and anticancer drugs including anti-CD20 monoclonal antibodies and several other molecular targeting drugs. Furthermore, it is speculated that the CD20-negative transformed phenotype may correlate with multi-drug resistance in the clinical setting. Re-biopsy of RD/PD tumors is helpful to define the patient disease condition and predict efficacy of monoclonal antibody therapeutics. Molecular analyses to determine additional genetic/epigenetic abnormalities at the RD/PD period correlated with CD20-negative phenotype may also be needed to ascertain the mechanisms of the CD20-negative phenotype and multi-drug resistance to develop strategies for overcoming refractory diseases.

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CONFLICT OF INTEREST DISCLOSURE

A.T. has no relevant conflicts to disclose.

REFERENCES

12 Tedder TF, Distech CM, Louie E, Adler DA, Croce CM, et al.: The gene that encodes the human CD20 (B1) differentiation antigen is located on chromosome 11 near the (t(11;14)(q13;q32) translocation site. J Immunol 142:2555-2559, 1989
19 Golay JT, Clark EA, Beverley PC: The CD20 (Bp35) antigen is involved in activation of B cells from the G0 to the G1 phase of the cell cycle. J Immunol 135:3795-3801, 1985
40 Nakamaki T, Fukushima K, Nakashima H, Arizumi H, Maeda T,
Genetic/epigenetic modulation of CD20 in B-cell malignancies


