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Review Article

Genetic and Epigenetic Modulation of CD20 Expression in B-Cell Malignancies: Molecular Mechanisms and Significance to Rituximab Resistance

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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 manuscript, 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

INTRODUCTION

After the introduction of rituximab, the first human anti-CD20 monoclonal mouse-human chimeric antibody,[1,2] into the clinical setting, the prognosis of most CD20-positive B-cell malignancies, including diffuse large B-cell lymphoma (DLBCL),[3,4] follicular lymphoma (FL)[5-7] and others, has been significantly improved. However, relapsed/refractory and progressive disease (RD/PD), even after performing combination chemotherapy with rituximab, is still a significant problem.[8-10] It was recently reported that aberrant expression of CD20 protein may play an important role for rituximab resistance in certain cases, especially after combination chemotherapy with rituximab. In this review, the molecular basics of *Membrane spanning 4-domains A1 (MS4A1; CD20)* gene expression and CD20 protein are introduced, and speculations about rituximab resistance from a viewpoint of aberrant CD20 expression through genetic and epigenetic mechanisms will be explained.

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MOLECULAR CHARACTERISTICS OF *MS4A1* GENE AND CD20 PROTEIN

The *MS4A1* gene is located on chromosome 11q12 and encodes CD20 (B1) protein.[11] The main transcript of the *MS4A1* gene is a 2.8 kb mRNA, and some transcript variants have also been identified.[12,13] CD20 is a transmembrane protein that has 2 extracellular domains (small and large loops) and 4 trans-membrane domains (Fig. 1A). The *MS4A1* gene has 8 exons, and the small and large extracellular domains are encoded mainly by exons 4 and 6, respectively (Fig. 1B). CD20 is a differentiation-related cell surface phosphoprotein (33, 35 and 37 kDa) that is expressed during early pre-B cell development just before the expression of cytoplasmic H chains and persists until plasma cell differentiation.[11,14,15] As CD20 protein is not expressed on hematopoietic stem cells or plasma cells, CD20 is a good molecular target for the treatment of mature B-cell malignancies by using anti-CD20

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.[12,16,17] Binding of anti-CD20 monoclonal antibodies with CD20 induces cell cycle arrest,[18] differentiation block or B-cell activation[19] through phosphorylation of CD20,[20] lipid raft localization of CD20 on cell membrane[21,22] and inducing intracellular calcium flux.[23] The responses of monoclonal antibodies are different depending on the antibodies used[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).[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,[25] which recognized the B-cell specific antigen L26. Anti-L26 antibody appeared to recognize the intracellular domain of CD20,[26] and is now widely utilized for immunohistochemistry (IHC) analyses in the clinical setting.

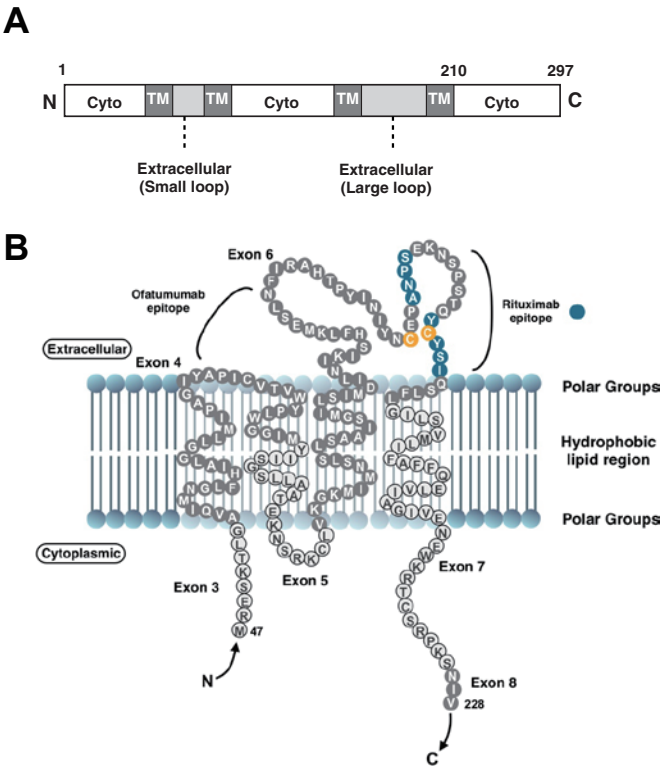

Fig. 1. Structure of CD20 protein. (***1A***) The *MS4A1* gene encodes CD20 protein (main product; 297 amino acids), that has 4 transmembrane domains (TM), 3 cytoplasmic domains (Cyto) and 2 extracellular domain (small and large loops). Shorter forms by splicing variants are also reported.[12,13] (***1B***) CD20 is a 4-transmembrane protein. Rituximab recognizes the 3D structure of the large extracellular loop (amino acids in the blue circle).[91] The disulfide bridge between cysteine C167 and C183 is indicated as C in the yellow circles. Ofatumumab recognizes the 3D structure of part of the small and large extracellular loops.[92] Amino acids encoded by exon 3 to 8 of the *MS4A1* gene are distinguished by white and gray circles. These figures were adopted from reports by Tedder *et al*.,[12] Binder *et al.*,[91] and Du *et al*.,[92] and modified. N and C; N- and C-terminus.

CD20 EXPRESSION IN B-CELL MALIGNANCIES

CD20 protein expression is detected in most B-cell malignancies.[15] However, the expression level is varies in each patient, even in those with the same diagnosis.[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 malignances (Fig. 2).[27] Miyoshi *et al*.[28] reported that CD20 protein expression confirmed by IHC and FCM in B-cell malignancies shows wide variation among patients. *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.[33-35] *De novo* DLBCL showing the specific phenotype of CD20 IHC-positive, but FCM-dim~negative (IHC+/FCM-), was also reported by several independent groups.[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.[29] Prognosis of this DLBCL phenotype by chemo-immunotherapy is still controversial.[29,30,33]

Aberrant down-modulation of CD20 protein expression in B-cell malignancies after rituximab use[10,28,30,36-44] and other

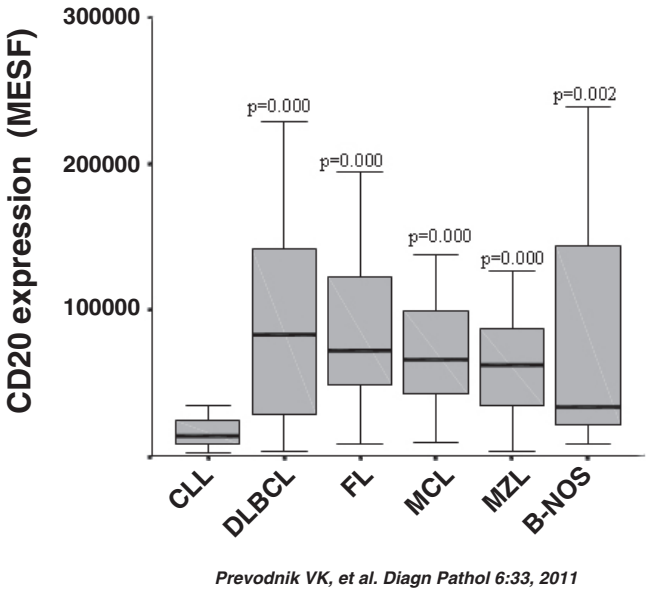

Fig. 2. CD20 protein expression on tumor cells of B-cell malignancies. Expression level of CD20 surface antigen was analyzed by flow cytometry and evaluated as mean fluorescent intensity (MFI). Note that CD20 protein expression was significantly lower in chronic lymphocytic leukemia than in other B-cell malignancies. This figure was adopted from a report by Prevodnik *et al.*.[27]

targeting drugs[45-47] has been observed in the clinical setting and also *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. Acquirement 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,[21,22,48] followed by signal transduction into the cytoplasm and calcium flux from the intracellular calcium pool.[23] After binding of rituximab, the complement complex is recruited to rituximab to form a membrane attack complex, and complement dependent cytotoxicity (CDC) occurs.[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.[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 cording region of the *MS4A1* gene,[28,40,51] aberrant expression of splicing variants of *CD20* mRNA,[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 *MS4A1* gene

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

	CD20 expression modulating factors	Putative mechanisms of CD20 expression modulation	References
Down-modulation	**Type I antibodies**		
	Rituximab	•Down-regulation of *MS4A1* gene expression by epigenetic mechanisms •Internalization of CD20 molecule •Selection of CD20-negative clones •Shaving of CD20/rituximab complex from cell surface	Tomita 2007, Hiraga 2009, Sugimito 2009, Beers 2010, Beum 2006, Williams 2006
	Molecular targeting therapeutics		
	Ibrutinib	•Inhibition of NFκB related factors that is critical for CD20 expression	Skarzynski 2015
	Lenalidomide	•Internalization of CD20 molecule	Lapalombella 2008
	Bortezomib	•Protein degradation by lysozomal/autophagic mechanisms	Bil 2010
	Genetic mutations		
	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	Terui 2009, Mishima 2011, Johnson 2009, Nakamaki 2012
	NOTCH1 mutation	•Recruitment of mutated-NOTCH1-RBPJ-HDAC complex to *MS4A1* gene promoter	Pozzo 2016
	Others		
	Smad2/3 expression	•Recruitment of Smad complex by TGFβ signal induce *MS4A1* gene repression	Kawabata 2012
	Splicing valiant	•Expression of the short form of CD20 that lacks exons 3 to 7.	Henry 2010
Stimulation	**HDAC inhibitors**		
	Trichostatin Valproic acid Rhomidepsine	•De-repression of *MS4A1* gene promoter by inhibiting HDACs recruited by transcription factors •Stimulation of expression of *MS4A1* targeted transcription factors*	Tomita 2007, Sugimito 2009, Shimizu 2010, Granata 2013, Damm 2015
	Demethylating agents (DNMT inhibitors)		
	Azacitidine Decitabine	•De-repression of *MS4A1* gene through CpG de-methylation induced by DNMTs** •Stimulation of expression of *MS4A1* targeted transcription factors*	Hiraga 2009, Sugimoto 2009, Granata 2013
	Other drugs		
	Farnesyltransferase inhibitors (L-744, 832)	•Recruitment of Pu.1/Oct2 transcription factors to *MS4A1* gene promoter	Winiarska 2012
	CpG oligodeoxynucleotides	•Stimulation of *MS4A1* gene expression by other than induction of Pu.1	Mankai 2009
	TGFβ inhibitor (LY364947)	•Inhibition of Smad2/3 recruitment to *MS4A1* gene promoter	Kawabata 2012

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 *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.[10,36,38,41,52] Furthermore, internalization or degradation of CD20 protein after treatment with several targeting drugs,[46,47,53] and shaving of the CD20-rituximab complex from the tumor cell surface by monocytes[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.[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 ([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.[57-61] Less effective ADCC activity due to Fc receptor polymorphisms is reported as a host condition.[62,63] As many genetic mutations have been recently reported in B-cell malignancies,[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 cording regions of the *MS4A1* gene were identified. Terui *et al.*[51] 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.*[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.*[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.*[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.*[31] 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

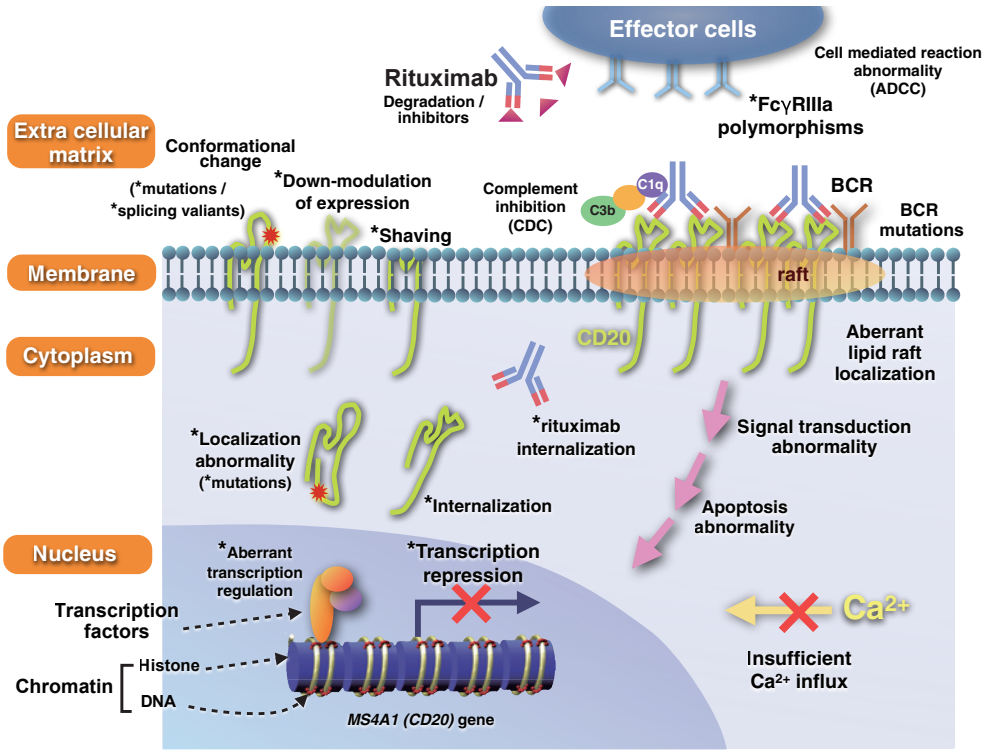

Fig. 3. Putative mechanisms of CD20 expression modification and rituximab resistance. Several mechanisms that likely contribute to aberrant CD20 expression are depicted. CD20 is indicated with a green line. The CD20 protein expression, structure and localization can be modified genetically and epigenetically,[10,38,52] *e.g.* splicing variants,[11,13] genetic abnormalities, such as mutations[51,68] and loss,[40] digestion (shaving) by monocytes[54] and internalization induced by several therapeutics[45,46] (also see Table 1). Abnormality of lipid raft localization of CD20 after binding with type 1 antibodies, including rituximab,[53] is also speculated. Insufficient antibody dependent cell mediated cytotoxicity activity by FcγRIIIa polymorphisms of patients[62] and apoptosis abnormalities by insufficient Ca^{2+} influx[23] are also speculated. Molecular mechanisms that were confirmed in clinical samples from rituximab resistant patients are indicated with asterisks (*).

into CD20-negative DLBCL after rituximab have been evaluated (Fig. 4A).[10,37-41,52,69] 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.

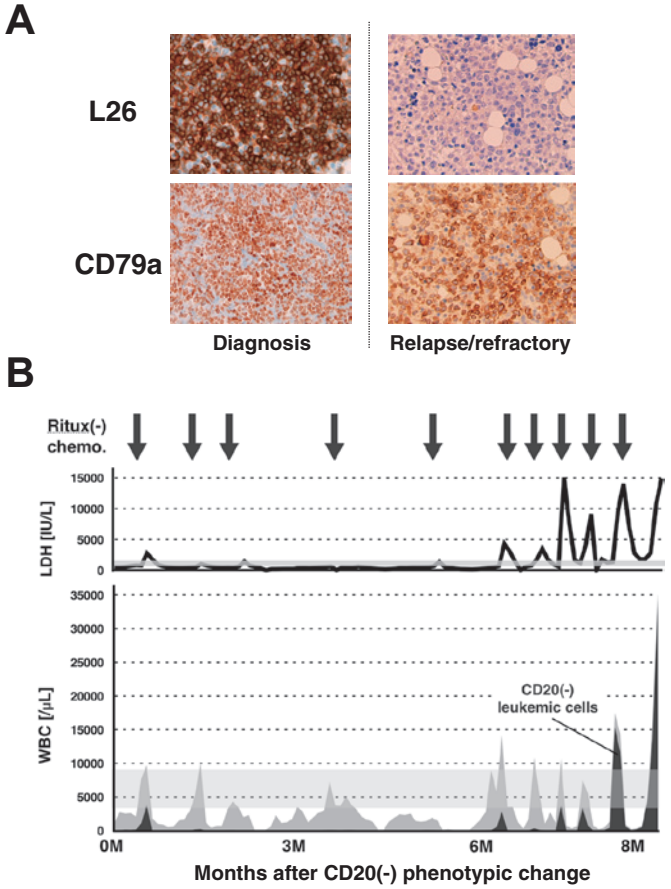

Fig. 4. Diffuse large B-cell lymphoma (DLBCL) showing CD20-negative phenotypic change after combination chemotherapy with rituximab. (***4A***) An example of a DLBCL patient who showed CD20-negative change after rituximab use is shown. Immunohistochemistry (IHC) showed L26 $(CD20)^+$ and $CD79b^+$ phenotype at diagnosis, but CD20 expression was not confirmed at relapsed/refractory and progress disease (RD/PD) period after chemotherapy with rituximab. (***4B***) A typical clinical course of a patient who exhibited CD20-negative phenotypic change[38] is indicated. A fifty-six year old man, who was diagnosed as follicular lymphoma grade 1-2 and received combination chemotherapy with rituximab. RD/PD with DLBCL phenotype (histological transformation) was confirmed after using rituximab repeatedly. The disease progression occurred with marked serum lactate dehydrogenase elevation and leukemic stage with infiltration of CD20-negative lymphoma/leukemia cells in peripheral blood at the terminal stage, despite salvage chemotherapies without rituximab (*black arrows*). He died of disease progression 8 months after confirming CD20-negative RD/PD. Lactate dehydrogenase level is indicated with the *black line*, and white blood cell count and CD20-negative leukemic cell count are indicated in *gray and black areas*, respectively. Detailed information about this patient was reported previously.[10,38]

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 phenotype[10] 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 monocytes[54] were reported (Table 1). Clonal selection of B-cell lymphoma cells with low-CD20 expression because of heterogeneous genetic backgrounds in each lymphoma cell in a patient may also be a reasonable mechanism of CD20-negative relapse after rituximab use.

To date, 3 cell lines from CD20-negative transformed patients after rituximab use have been established (RRBL1,[38] WILL2[39] and SD07[40]), and molecular mechanisms have been analyzed. RRBL1 cells are the cell line established from peripheral blood CD20-negative tumor cells of a patient

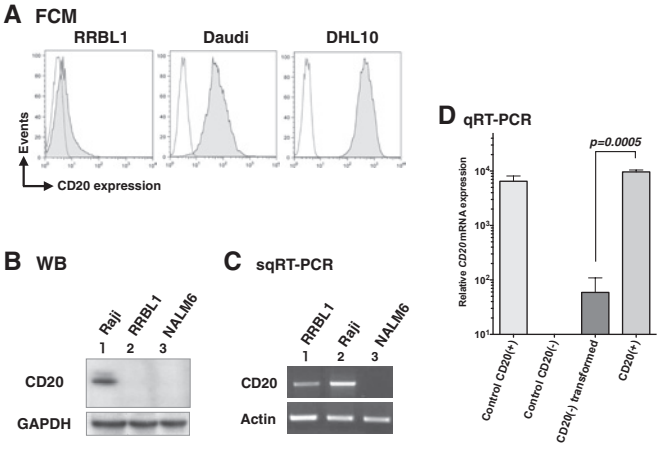

Fig. 5. Molecular background of lymphoma cells with CD20-negative phenotypic change. RRBL1 cells were established from peripheral blood tumor cells from the patient indicated in Fig. 4B, and used for molecular analysis. (***5A***) Flow cytometry (FCM) analysis using anti-CD20 antibody (B2E9) was performed for B-cell lymphoma/leukemia cell lines. Note that CD20 protein expression (*shaded area*) in RRBL1 cells was significantly low compared with CD20-positive Daudi and DHL10 cells (*solid line*; isotype control). CD20 protein and mRNA expression was confirmed by (***5B***) Western blotting (WB) using anti-CD20 antibody and (***5C***) semi-quantitative real-time polymerase chain reaction (RT-PCR).[38] Raji and NALM1 cells were used for positive and negative controls. (***5D***) *CD20* mRNA expression was confirmed by quantitative RT-PCR using CD20-positive and -negatively transformed clinical samples.[29]

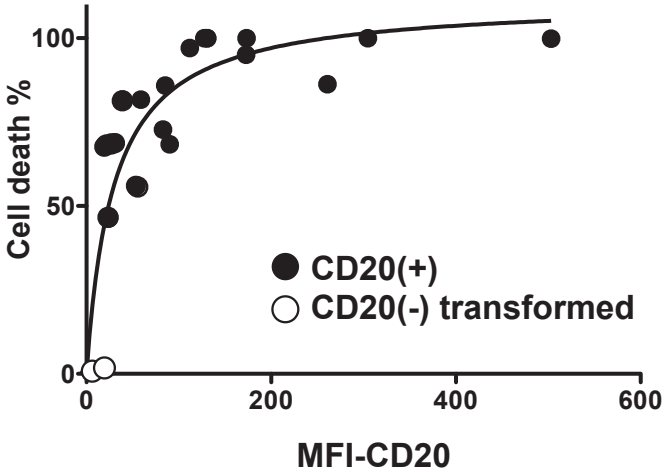

Fig. 6. CD20 protein expression level and rituximab sensitivity. *In vitro* complement dependent cytotoxicity (CDC) assay was performed as previously reported.[29] Relationship between the mean fluorescent intensity in flow cytometry using anti-CD20 antibody (B2E9) and rituximab induced cell death percentage in the *in vitro* CDC assay is indicated. Note that CD20 protein expression level is critical for rituximab induced CDC activity.

indicated in Fig. 4B. CD20 protein expression in both FCM and immunoblotting analyses was significantly lower in RRBL1 than in typical CD20-positive B-cell lines (Fig. 5A & 5B). Semi-quantitative and quantitative real-time polymerase chain reaction (RT-PCR) indicated that although *CD20* mRNA expression was almost 100-times lower in RRBL1 cells (also WILL2 cells[29,39]) 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).[10,29] 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)[75] and 5-aza-deoxycitidine (5-Aza-dC)[10,52]] and histone deacetylase (HDAC) inhibitors (trichostatin A (TSA),[38] valproic acid[75-77] and romidepsin[76]) were evaluated *in vitro* and *in vivo* 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[38] and 5-Aza-dC,[10] and the efficiency was enhanced by combination of these two drugs (Fig. 7A).[52] Molecular analyses indicated that DNMT1 depletion occurred after administration of 5-Aza-dC as previously reported,[78] 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 to be the reason for *MS4A1* gene up-regulation.[52] 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[52] (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.*[76] 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.*[79] 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 ALL[75] and CD20-positive DLBCL.[77] In these reports, CD20 protein expression level was moderately up-regulated 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.

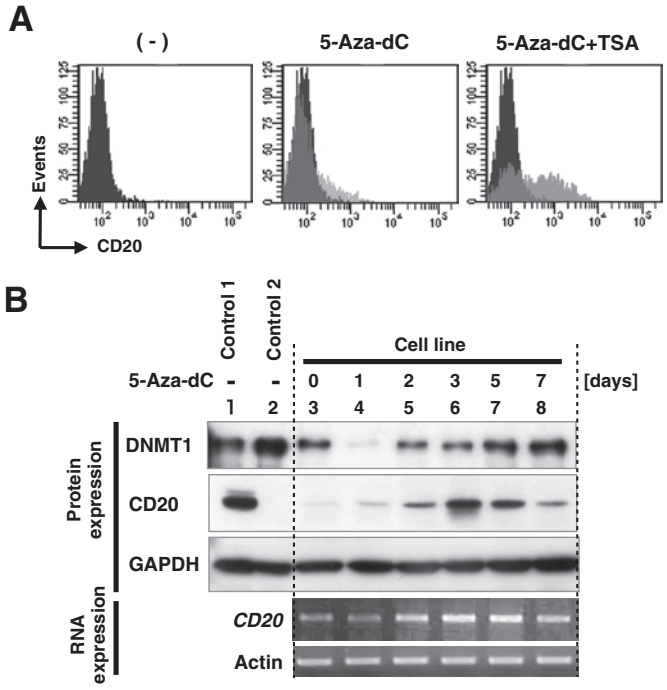

Fig. 7. Epigenetic regulation of *CD20* mRNA and protein expression. (***7A***) RRBL1 cells were treated with 5-aza-deoxycitidine (5-Aza-dC), a DNA methyltransferase (DNMT) inhibitor, and trichostatin A (TSA), a histone deacetylase inhibitor. CD20 protein expression was confirmed by flow cytometry. (***7B***) 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.

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) mutation[82] 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

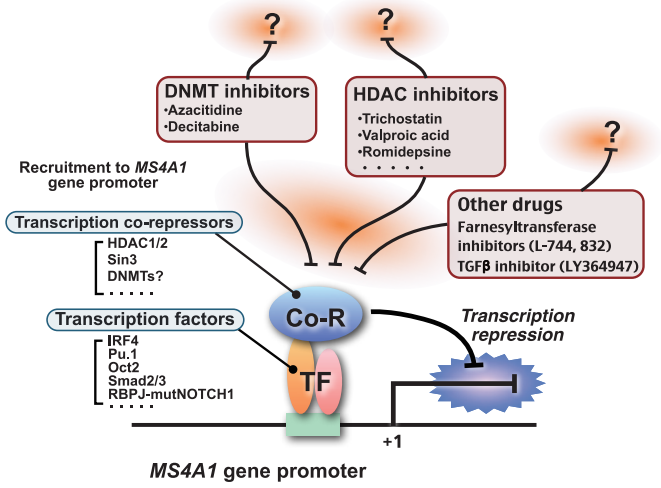

Fig. 8. Mechanisms of *CD20* mRNA down-regulation through transcription factors and transcription co-repressors (Co-R). Several transcription factors, such as IRF4, Pu.1 and Oct2, are recruited to the *MS4A1* gene promoter and may contribute to transcription repression in the presence of Co-R.[52,80,93] In few situations, Smad2/3[83] and RBPJ-mutated-NOTCH1[56] 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 TGFb signaling pathway was negatively correlated with CD20 expression in B-cell non-Hodgkin lymphoma.[83] They demonstrated that in Ramos cells, Smad2/3 were recruited to the *MS4A1* promoter in the presence of TGFb to repress transcription, and *MS4A1* gene expression was stimulated by the TGFb inhibitor, LY364947.

Several reports demonstrated that CD20 expression can be modulated by molecular targeting drugs. Beers *et al*.[53] reported that CD20 on B-cell malignancies internalized after binding with type I anti-CD20 monoclonal antibodies (rituximab-like),[22] resulting in reduced macrophage recruitment and degradation of CD20/antibody complexes. This phenomenon cannot be observed when using type II antibodies (tositumomab-like), and was highly observed in CLL and mantle cell lymphoma compared with in FL or DLBCL. CD20 protein internalization was also observed[46] when lenalidomide, one of the celebron-interacting immunomodulatory drugs,[84] was used on primary CLL cells without influencing *MS4A1* transcription. Bil *et al.*[47] 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 lenelidomide[85,86] and bortezomib,[87,88] especially for non-germinal center B-cell type (activated B-cell type) DLBCL,[89,90] was recently reported, CD20 protein expression level and rituximab sensitivity in rituximab-combination therapy should be evaluated in the future. Skarzynski *et al.*[45] reported that ibrutinib, a Bruton tyrosine kinase (Btk) inhibitor that leads to NFkB pathway down-regulation, decreased CD20 protein expression on CLL cells in the clinical setting. They speculated that the NFkB consensus sequence was in the *MS4A1* gene promoter, thus, inhibition of the NFkB 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.

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