Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance

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Running title: Loss of CD20 expression after rituximab treatment

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Abstract

Although rituximab is a key molecular targeting drug for CD20-positive B-cell lymphomas, resistance to rituximab has recently been recognized as a considerable problem. Here we report that a CD20-negative phenotypic change after chemotherapies with rituximab occurs in a certain number of CD20-positive B-cell lymphoma patients. For 5 years, 124 patients with B-cell malignancies were treated with rituximab-containing chemotherapies in Nagoya University Hospital. Relapse or progression was confirmed in 36 patients (29.0%), and a re-biopsy was performed in 19 patients. Of those 19, 5 (26.3%) (DLBCL; 3 cases, and DLBCL transformed from FL; 2 cases) indicated CD20 protein-negative transformation. Despite salvage chemotherapies without rituximab, all 5 patients died within 1 year of the CD20-negative transformation. Quantitative RT-PCR showed that CD20 mRNA expression was significantly lower in CD20-negative cells than in CD20-positive cells obtained from the same patient. Interestingly, when CD20-negative cells were treated with 5-aza-2'-deoxycytidine in vitro, the expression of CD20 mRNA was stimulated within 3 days, resulting in the restoration of both cell surface expression of the CD20 protein and rituximab sensitivity. These findings suggest that some epigenetic mechanisms may be partly related to the down-regulation of CD20 expression after rituximab treatment.
Introduction

Rituximab is a murine/human chimeric-anti-CD20 monoclonal antibody that has become a key molecular-targeting drug for CD20-positive B-cell lymphomas\textsuperscript{1,2}. Many favorable results using combination chemotherapy with rituximab for both CD20-positive \textit{de novo}- and relapsed-low grade and aggressive B-cell non-Hodgkin’s lymphoma have been reported in recent years\textsuperscript{3-7}. In Japan, rituximab has also been utilized since September 2001 for patients with follicular lymphoma (FL), indolent lymphoma, and mantle cell lymphoma (MCL). Also, since September 2003 in Japan, indications for utilizing rituximab were expanded to include diffuse large B-cell lymphoma (DLBCL), further demonstrating the significant effectiveness of rituximab for B-cell lymphoma compared to conventional chemotherapies without rituximab\textsuperscript{8}.

Although combination chemotherapies with rituximab have provided significantly favorable results for CD20-positive B-cell lymphoma patients, acquired resistance to rituximab has become a considerable problem. Several mechanisms of resistance were predicted as reported previously, including loss of CD20 expression, inhibition of antibody binding, antibody metabolism, expression of complement inhibitors such as CD55/CD59, and membrane/lipid raft abnormality\textsuperscript{10-19} (reviewed by Smith \textit{et al.}\textsuperscript{9}), but the clinical significance of those mechanisms has remained unclear. In the last 5 years, a CD20-negative phenotypic change in CD20-positive lymphomas after rituximab treatment has been reported by several groups\textsuperscript{16,20-31}, indicating that this phenomenon following the use of rituximab may not be rare. Although these reports contain important information from clinical experiences, the frequency of occurrence and detailed molecular biological information about the CD20-negative phenotype remain to be elucidated.

Very recently, we reported a CD20-negative DLBCL case that had transformed from CD20-positive FL after repeated treatment with rituximab. We established an RRBL1 cell line from this patient\textsuperscript{32}, and the mechanisms of the CD20-negative change were analyzed in these cells. \textit{CD20} mRNA expression was significantly lower than in CD20-positive cells, resulting in a loss of CD20 protein expression as detected by flow cytometry (FCM), immuno-histochemistry (IHC), and immunoblotting (IB). Interestingly, trichostatin A (TSA), a histone deacetylase inhibitor, was able to
successfully stimulate CD20 expression, suggesting that some epigenetic mechanisms may have repressed the expression. Thus, an accumulation of detailed clinical and molecular biological features is required to demonstrate the significance of CD20-negative phenotypic changes after rituximab treatment.

In the last 5 years, 124 patients with CD20-positive B-cell malignancies received chemotherapy with rituximab at Nagoya University Hospital, 36 of whom showed relapse/progression. Among those 36, CD20 protein-negative or -decreased phenotypic changes were confirmed in 5 cases concomitant with disease progression. Here we describe the occurrence rate of CD20-negative transformation after rituximab treatment, and the molecular background of the CD20 protein-negative phenotype in cells from those patients.

Methods

Patients
Between February 1988 and November 2006 in Nagoya University Hospital, Nagoya, Japan, all 124 patients in this analysis were initially diagnosed with CD20-positive B-cell lymphomas (Table 1) according to the World Health Organization (WHO) classification. All patients were treated with combination chemotherapy with rituximab from September 2001 to December 2006. The median age of the patients was 58 years (range; 16-84) at the time of initial rituximab administration. Three patients had received rituximab before September 2001 because of their participation in a previous clinical study. The most recent follow-up date was July 31, 2007, and disease status factors such as relapse, recurrence, and progression were determined by clinical findings and diagnostic imaging using X-ray photography, computed tomography (CT), magnetic resonance imaging (MRI), and 18F-fluorodeoxyglucose positron emission tomography (FDG-PET). Re-sampling of tumors at the time of relapse/progression and pathological analysis of 19 patients was performed. The patients’ response to chemotherapies was evaluated using the International Working Group criteria.
Confirmation of CD20 protein expression by IHC and FCM analyses

These studies were conducted with institutional review board approval from the Nagoya University School of Medicine. After obtaining appropriate informed consent from each patient, in accordance with the Declaration of Helsinki, tumor specimens were harvested from lymph nodes, bone marrow, peripheral blood, or spinal fluid. CD20 protein expression was demonstrated by IHC and/or FCM as indicated previously. Briefly, we used mouse anti-CD20 (L26) (Dako, Carpinteria, CA, USA), anti-CD10 (Novocastra, Newcastle upon Tyne, UK), and anti-CD79a monoclonal antibodies (Dako) for IHC, and mouse anti-CD20 (B2E9) (Beckman Coulter, Fullerton, CA, USA) and anti-CD19 (HD37) (Dako) monoclonal antibodies for FCM. The CD79a antigen is a pan-B-cell marker that forms a B-cell receptor (BCR) protein complex. The percentage of negative and positive cells from FCM was determined from the data using an isotypic control antibody (mouse IgG1) (Beckman Coulter).

Sequence analysis of the MS4A1 (CD20) gene

Genomic DNA from tumor cells was extracted with a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA), and used for further polymerase chain reactions (PCR). When sufficient tumor cells could not be obtained at diagnosis, genomic DNA from paraffin sections was extracted using the Magnesil Genomic Fixed Tissue System (Promega, Madison, WI, USA). Genomic DNA PCR was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) to acquire fragments of the coding sequences of exons 3 to 8 of the MS4A1 (CD20) gene. The following primers were designed from the appropriate intron sequences to achieve the coding sequences: exon 3-U, 5’-GCT CTT CCT AAA CAA CCC CT-3’, exon 3-L, 5’-CAT GGG ATG GAA GGC AAC TGA C-3’, exon 4-U, 5’-TGC TGC CTC TGT TCT CTC TCT TCT CTG TCT CC-3’, exon 4-L, 5’-CTG CAC CAT TTC CCA AA T GGC T-3’, exon 5-U, 5’-CTC CA T CTC CCC CAC CTC TC-3’, exon 5-L, 5’-GGT ACT TCT CTG ACA TGT GGG A-3’, exon 6-U, 5’-TGG AAT TCC CTC CCA GA T TA T G-3’, exon 6-L, 5’-CCT GGA GAG AAA TCC AAT CTC A-3’, exon 7-U, 5’-GTT ACT TCT CTG ACA TGT GGG A-3’, exon 7-L, GGC TAC TAC TTA CAG ATT TGG G-3’, exon 8-U, 5’-TGG TCA ATG TCT GCT GCC CT-3’, and exon 8-L, 5’-GCG TAT GTG CAG AGT ACC TCA AG-3’. Amplified fragments were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA) and
were sequenced using a DNA auto sequencer (ABI prism 310, Applied Bio Systems). PCR fragments that contained each exon sequence were cloned into the pGEM-T vector, and at least 10 clones were sequenced. If a mutation was observed in 2 different clones, we verified that the sequence reflected a mutation in the tumor rather than a PCR error.

**RNA extraction and reverse transcriptase-PCR (RT-PCR)**

A Blood RNA Extraction Kit (Qiagen) was used to isolate total RNA from tumor cells. Complementary DNA (cDNA) was prepared as reported previously. For RT-PCR analyses of CD10, CD19, CD20, and beta-actin, the following primers were designed: CD10-U, 5′-TTG TCC TGC TCC TCA CCA TC-3′, CD10-L, 5′-GTT CTC CAC CTC TGC TAT CA-3′, CD19-U, 5′-GAA GAG GGA GAG GAT AAC GCT GT-3′, CD19-L, 5′-CTG CCC TCC ACA TTG ACT G -3′, CD20-U, 5′-ATG AAA GGC CCT ATT GCT ATG-3′, CD20-L, 5′-GCT GGT TCA CAG TTG TAT A TG-3′, beta-actin-U, 5′-TCA CTC AAG ATC CTC A-3′, and beta-actin-L, 5′-TTC GTG GAT GCC ACA GGA C-3′.

Semi-quantitative RT-PCR with AmpliTaq Gold was performed as described previously. Quantitative-RT-PCR was carried out using TaqMan PCR (ABI prism 7000, Applied Biosystems, Foster City, CA, USA) as previously described.

**Immunoblot analysis**

Cells (~5 X 10^5) were lysed in 100 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 1.5 mM MgCl2, 1 mM EGTA, 5 mM KCl, 10% glycerol, 0.5% NP-40, 300 mM NaCl, 0.2 mM PMSF, 1 mM DTT, and a complete-mini protease inhibitor tablet (Roche)). After centrifugation at 10,000 Xg for 10 minutes, the supernatants were placed in new tubes and 100 μl of 2X SDS sample buffer was added. After boiling for 5 minutes, samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was carried out as described previously using goat polyclonal anti-CD20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and rabbit polyclonal anti-actin antibody (Santa Cruz Biotechnology).

**Treatment of RRBL1 cells and primary lymphoma cells with the epigenetic drug 5-aza-2′ deoxycytidine**
RRBL1 and primary lymphoma cells (5X10^5) were cultured in 6-well dishes in RPMI medium containing 10% FBS for 24 hours with or without 5-aza-2′ deoxycytidine (5-Aza) (Sigma, St. Louis, MO) at a final concentration of 100 mM. The cells were then washed twice with RPMI medium containing 10% FBS, and incubated for more than 2 days in the same medium without 5-Aza. Cells were harvested and utilized for total RNA and protein extraction.

**Antibody-dependent cell-mediated cytotoxicity (ADCC) assay in vitro**

ADCC activity was analyzed by an in vitro chromium-51 (\(^{51}\)Cr) release assay. Target cells (RRBL1, Daudi, DHL10) were cultured in appropriate medium supplemented with 10%-20% FBS. Each cell line (2.0 x 10^5 cells) was labeled with 3.7 MBq of Na\(_2\)\(^{51}\)CrO\(_4\) (Perkin Elmer Japan, Tokyo, Japan) at 37°C for 1 hour. Human peripheral blood mononuclear cells (PBMC), which were obtained from a healthy donor, were prepared as effector cells of the cell-mediated cytotoxicity assay. Chromium-51- labeled target cells were aliquotted into 96-well plates (1 x 10^4 cells/well). Effector PBMC cells (5 x 10^5 cells/well) were then added to each well in the presence or absence of rituximab (0 to 31.25 \(\mu\)g/ml), and incubated for 4 hours at 37°C. Supernatants were obtained after a brief centrifugation, and measured on a gamma-ray counter (Perkin Elmer). Chromium-51-labeled target cells without antibodies were lysed completely by NP-40 (2% final concentration), and utilized as a positive control (the maximal \(^{51}\)Cr release). The percentage of lysed cells was calculated using the following formula: % cell lysis = (experimental release (cpm) – background (cpm)) / (maximal release (cpm) – background (cpm)) x 100%.

**Results**

**CD20-negative phenotypic change after treatment with rituximab**

A total of 124 patients with CD20-positive B-cell malignancies were treated with rituximab-combined chemotherapy from September 2001 to December 2006 (Table 1).
All patients were diagnosed with CD20-positive B-cell lymphomas by IHC and/or FCM analyses using their tumor tissue specimens. Thirty-six patients (29.0%) showed relapse and progression (response; RD/PD in Table 1) of their disease after or during chemotherapies with rituximab. Tumor cells from 19 of these 36 patients (52.8%) were re-sampled at the time of RD/PD, and CD20 protein expression was analyzed by IHC and/or FCM. CD20 protein expression was not detected or was significantly decreased in 5 patients (DLBCL; 3 patients, FL; 2 patients). Therefore, in 26.3% of patients whose tumor cells were re-sampled at the time of RD/PD, a CD20-negative phenotypic transformation after rituximab treatment was observed.

**Clinical and laboratory features of patients with a CD20-negative phenotypic change**

The clinical features of the 5 patients with a CD20-negative phenotypic change after rituximab treatment are shown in Table 2. Initially, 3 patients were diagnosed with DLBCL and 2 patients were diagnosed with FL. They were treated with chemotherapy with rituximab (375 mg/m²) repeatedly until a CD20-negative phenotypic change was observed. Four to 14 cycles of rituximab were administered. These 5 patients showed relapse or progression from 2 to 81 months after their first treatment with rituximab. Histological transformation from FL was observed in 2 patients, resulting in all 5 patients being diagnosed histologically as DLBCL at the time of RD/PD. Tumor cell infiltration into the bone marrow was observed in all 5 patients. A CD20 protein-negative phenotype was confirmed by IHC (Figure 1) and FCM in all 5 cases. In 3 patients, mRNA from tumor tissues was available, and CD20 mRNA expression was faintly observed using RT-PCR. Although all 5 patients received salvage chemotherapy without rituximab, they all died from disease progression within 11 months of the confirmation of CD20-negative transformation. The clinical outcomes of these patients who showed RD/PD after treatment with rituximab-containing chemotherapy are shown in Table 3. The 5 patients with CD20-negative RD/PD tended to have a shorter survival time than with CD20-positive RD/PD (100% vs. 35.7% died). However, statistical significance could not be determined because of the variable disease status of each patient, including different backgrounds, salvage chemotherapies, etc. More patients must be studied to further analyze this apparent trend.
Genetic abnormalities in the *CD20* gene

Genomic DNA mutations in the coding sequence (CDS) of the *CD20* gene (also known as the *MS4A1* gene) were also analyzed in the 5 patients. If the mutations were located in specific domains that are recognized by anti-CD20 antibodies including rituximab, those mutations might be related to resistance to rituximab and/or the CD20-negative phenotype. As indicated in Table 2, the change in serine 97 to phenylalanine (S97F; TCC -> TTC) in UPN #1 and valine 247 to isoleucine (V247I; GTT -> ATT) in UPN #2 were confirmed in 2 clones each out of 16 and 10 clones, respectively. In the other 3 cases, no genetic mutations in the *MS4A1* CDS were detected. Chromosomal analysis by G-banding was also performed using tumor cells obtained from each patient in both the initial diagnosis (CD20-positive) and at the time of RD/PD (CD20-negative) (supplemental table). Chromosomal abnormalities involving 11q12 containing the *MS4A1* gene were not observed.

Alteration of CD20 protein and mRNA expression levels after treatment with rituximab

As shown in Figure 1, CD20 protein expression by IHC analysis is altered after chemotherapy with rituximab. The clinical course of UPN #3 is depicted briefly in Figure 2A. FCM analyses using appropriate lymphoma tissues from lymph nodes, peripheral blood, bone marrow, and/or cerebral fluid were performed at admission (I), upon relapse after treatment with rituximab-containing combination chemotherapy (II), and at the end stage of disease after salvage chemotherapy without rituximab (III). The results of FCM analysis using peripheral blood, which contains lymphoma cells, are shown in Figure 2B. Interestingly, CD20 protein expression recognized by FCM analysis was significantly diminished at time period II, but was reversed at III. At time period II, CD20-negative lymphoma cell infiltration into the cerebral fluid was also confirmed by FCM analysis (data not shown). On the other hand, CD19 expression, which is also present on B-cell lymphoma cells, was detected constantly throughout the clinical course (Figure 2B, right column). Semi-quantitative RT-PCR (Figure 3A) and quantitative RT-PCR (Figure 3B) show that the mRNA expression level of *CD20* was significantly altered in each stage. *CD20* mRNA expression was faintly observed at
stage II (Figure 3B, column 4) when CD20 protein expression was barely detectable with FCM (Figure 2B) and IHC (Figure 1C). In UPN #4, CD20 mRNA expression levels were determined by RT-PCR using tumor samples obtained before and after treatment with rituximab-containing chemotherapy (Figure 3C and D). Similar to UPN #3, CD20 mRNA expression was significantly decreased, and no protein expression was detected with IHC (Figure 1B). These findings suggest that CD20 protein expression is mainly regulated at the transcriptional level, and that the expression may be down-regulated in patients who show CD20-negative transformation after treatment with rituximab.

**Epigenetic regulation of CD20 gene expression after treatment with rituximab**

These findings suggest that CD20 expression is partly epigenetically regulated by factors such as rituximab treatment surrounding tumor cells, or that CD20-negative tumor cells are able to grow selectively during rituximab treatment. If the CD20-negative B-cells still possess the capability to express CD20 protein, we hypothesized that some epigenetic drugs may be able to stimulate the CD20 transcription. First, we examined CD20 transcription after treatment with 5-aza-2'-deoxyctidine (5-Aza) using primary tumor cells derived from cerebral fluid of UPN #3 at time period II in Figure 2A, which showed a CD20-negative phenotype. As a CD20-positive control, lymphoma cells at the initial diagnosis from the same patient were utilized (Figure 4A, lane 1). After treatment with 5-Aza in vitro, significant stimulation of CD20 expression was observed (lane 3).

Previously, we established the CD20-negative B-cell lymphoma cell line RRBL1, which was derived from CD20-negative tumor cells in peripheral blood from a patient (patient UPN #5 in Table 2). Next, we performed the same assay using these cells (Figure 4B), and we were able to show up-regulation of CD20 mRNA expression (Figure 4B and C). CD20 protein expression induction was also confirmed by immunoblotting (Figure 4B, IB). Thus, these data showing that CD20 expression could be stimulated within a few days suggested that CD20 expression is down-regulated by epigenetic mechanisms.

**Restoration of rituximab sensitivity in CD20-negative cells after treatment with 5-**
From these findings, we hypothesized that rituximab sensitivity would be restored if we could stimulate CD20 protein expression on the surface of CD20-negative transformed lymphoma cells. To test this hypothesis, we performed FCM analysis and an *in vitro* ADCC assay using RRBL1 cells with or without 5-Aza treatment. As shown in Figure 4D, CD20 protein expression was induced on the surface of 30.7% of RRBL1 cells after treatment with 5-Aza. Using these cells with or without 5-Aza treatment, an *in vitro* 51Cr-release assay was performed to confirm ADCC activity induced by rituximab (Figure 4E). Daudi cells were used as CD20-positive rituximab-sensitive control cells.

In the presence of rituximab, cell death was observed in a dose-dependent manner in Daudi cells. In contrast, the percentage of RRBL1 cells undergoing cell death was significantly lower despite the high concentration of rituximab. RRBL1 cells treated with 5-Aza showed partial rituximab sensitivity compared to RRBL1 cells not treated with 5-Aza. These experiments were done in triplicate and repeated at least 3 times, with similar results. These data suggest that CD20 expression and rituximab sensitivity could be restored in some cases using epigenetic drug treatment even when CD20-negative transformation results from rituximab treatment. Further experiments using patients’ primary samples and an *in vivo* system will be required to further explore this idea.

**Discussion**

Rituximab is a clinically important anti-tumor monoclonal antibody targeting the CD20 surface antigen expressed on B-cell malignancies. However, its effectiveness is sometimes unsatisfactory since a significant percentage of patients treated with rituximab-containing chemotherapy showed relapse or progression\(^{3,6,40}\). In this report, we also estimated that RD/PD after treatment with rituximab was observed in almost 30% of B-cell lymphoma patients after treatment with combination chemotherapies with rituximab. It is important to note that not all patients who show RD/PD demonstrate "rituximab resistance." In fact, some patients were sensitive to re-treatment with
rituximab-containing salvage chemotherapies (data not shown). We may need to define "rituximab resistance" more carefully by monitoring each individual's clinical course. A CD20-negative phenotypic change was observed in 26.3% of patients for whom tumor re-sampling (re-biopsy) was carried out (Table 1). We generally perform re-biopsies when tumor progression becomes very aggressive or when the manner of tumor expansion significantly changes during clinical observation. If we had carried out re-sampling on every RD/PD patient, the percentage of patients with CD20-negative transformations may have been much lower. Thus, examination of more patients will be critical. One important observation about the CD20-negative phenotypic change is that all five patients died from their disease progression within 1 year after showing a CD20-negative transformation (Table 2 and 3). This observation may indicate that a loss of CD20 expression is partly related to poor prognosis. In our study however, patients' backgrounds varied, e.g. age, sex, pathological findings, chemo-regimens, and organ function. A larger patient sample is warranted to determine the significance of the loss of CD20 expression.

It is noteworthy that CD20 mRNA expression was confirmed by RT-PCR even in those tumor cells that showed a CD20-protein-negative phenotype using IHC, FCM, and immunoblotting (Figure 3). In one case (Figure 3, UPN #3), expression of CD20 mRNA and protein were observed again after salvage chemotherapy without rituximab. Clonal evolution may be one reason for the alteration of CD20 mRNA and protein expression patterns in the same patient either with or without rituximab. However, our finding of the restoration of CD20 mRNA and protein expression within 3 days after treatment with 5-Aza (Figure 4), instead may support the idea that expression is regulated by epigenetic mechanisms, rather than by the alteration of several tumor clones.

We cannot exclude the possibility that genetic alteration in tumor cells that affects the expression of transcription factors Pu.1, Pip, and Oct2, which are thought to be critical for MS4A1 (CD20) gene expression⁴¹, may contribute to the aberrant CD20 transcriptional regulation. We analyzed the methylation status of CpGs in CD20 promoters almost 1000 bp upstream from the transcription start site to determine the mechanism of transcription up-regulation by 5-Aza treatment. Interestingly, CpG islands do not exist in the promoter site, and only 4 CG sequences can be observed in that region. Methylation of the 4 CG sites was not observed in the tumor cells from
UPN #5 and RRBL1 cells using bisulfite sequencing (data not shown). Mechanisms other than DNA methylation of the CD20 promoter may also be responsible for aberrant transcription down-regulation.

It is also possible that down-regulation of CD20 protein via such mechanisms as microRNA, protein folding, exportation, or glycosylation, may occur. A recent report suggests the possibility that both CD20 gene expression (at the pre- and post-transcriptional level) and protein down-regulation are related to the loss of CD20 protein expression after treatment with rituximab in vitro, resulting in rituximab resistance. Also, down-regulation of CD20 protein surface expression by internalization into the cytoplasm was also observed in some specific cases. Further molecular analysis of the down-regulation of CD20 protein after treatment with rituximab is needed.

Another interesting finding in our study is that all the patients showing a CD20-negative phenotypic change have been diagnosed as DLBCL. Two cases were diagnosed as FL at their first admission, but both were transformed into DLBCL when a CD20-negative change was observed. Furthermore, a CD20-negative change was confirmed in all 5 cases using tumor cells derived from the bone marrow and/or cerebral fluid. These findings may suggest that the clinical entity and progression pattern is partly related to CD20-negative phenotypic transformation. Further studies will be needed to confirm this idea.

Genetic mutations in the CD20 coding sequence were also observed in two cases, as shown in Table 2. These mutations lead to amino acid alterations including S97F and V247I, which are located at the second trans-membrane domain and the C-terminal intracellular domain, respectively. A recent report suggests that neither site is recognized directly by rituximab. Although it is possible that these alterations lead to a conformational change in the CD20 protein that interferes with rituximab binding, a more attractive explanation may be that the loss of expression is much more critical for resistance to rituximab than we originally suspected. Preliminary data using fluorescence-labeled rituximab indicate that rituximab fails to bind to RRBL1 cells (CD20-negative B-cells) in vitro (data not shown), and that ADCC and CDC (complement-dependent cytotoxicity) activity in vitro are significantly lower than in CD20-positive B-cells (CDC; data not shown). These data suggest that the loss of antibody binding due to the down-regulation of antigen expression is one critical
mechanism underlying rituximab resistance. Further investigation will be needed to expand these observations.

Our observations also revealed a population of cells that are rituximab resistant despite the presence of CD20 protein expression as observed by FCM, IHC, and immunoblotting (data not shown). In those patients, molecular mechanisms other than a loss of protein expression may have occurred, such as an amino acid alteration resulting from a genetic mutation of the MS4A1 gene, a post-translational modification of the CD20 protein, abnormalities in the CD20 signal transduction pathway, anti-apoptotic mechanisms of tumor cells, or aberrant metabolism of rituximab\textsuperscript{9,44,45}. The detailed mechanisms of these and other possibilities are still unclear.

Finally, in the specific cases reported here, 5-Aza can stimulate CD20 mRNA and protein expression, resulting in the restoration of rituximab sensitivity \textit{in vitro}. The DNA methyltransferase inhibitors 5-azacytidine and 5-aza-2’-deoxycytidine have been utilized in patients suffering from hematological malignancies such as myelodysplastic syndrome\textsuperscript{38,39,46}. In the future, a combination of molecular targeting therapy using 5-Aza and rituximab may prove to be a unique strategy as a salvage therapy for CD20-negative transformed B-cell malignancies in certain individuals. Further analysis of patients’ primary cells and \textit{in vivo} analysis using mouse xenograft lymphoma models are required.

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\textbf{Contributions of the authors}

Contribution: J.H and A.T. designed experiments, performed research, analyzed data, and wrote the paper; T.S. and K.S. prepared clinical samples and performed research;
M.I. and S.N. performed pathological analyses; H.K. and T.K. analyzed data, designed experiment and interpreted data; and T.N. supervised experiments and wrote the paper. Conflict-of-interest disclosure: H.K. is a consultant for a Kyowa Hakko Kogyo, Co. Ltd. The other authors declare no competing financial interests. T.K. is a consultant for Chugai Pharmaceutical Co., Ltd. and Zenyaku Kogyo Co., Ltd.

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Figure legends

Figure 1. CD20 protein-negative phenotypic changes in CD20-positive B-cell lymphoma patients after treatment with rituximab-containing chemotherapy. Tissue samples (LN, lymph nodes; CS, bone marrow clot section) obtained from UPN #1 (A), UPN #4 (B), and UPN #3 (C) in table 2 were analyzed by IHC using anti-CD20, -CD79a, and -CD10 antibodies. Anti-CD79a antibody was utilized for detection of B-cells. Note that CD20 was positive at the time of initial diagnosis in these patients, and that the CD20-negative phenotypic change was observed during the relapse/progression period. Original magnification, 400X (A) and 200X (B and C). HE, hematoxylin-eosin staining; RD/PD, relapsed/progression of disease.

Figure 2. Alteration of CD20 protein expression on B-cell lymphoma cells during disease progression. (A) The clinical course of UPN #3 is depicted briefly. Black and gray arrows indicate 1 course of combination chemotherapy with or without rituximab, respectively. Rituximab (375 mg/m² each) was administered 7 times. During the patient's 24 month clinical course, tumor cells were harvested at time periods I, II, and III from lymph nodes, bone marrow, peripheral blood, and/or cerebral fluid. (B) FCM analysis using anti-CD20 and -CD19 antibodies was carried out using tumor cells from peripheral blood. Positive cells are shown in the black lines, and the percentage of positive cells is shown. Note that CD20 expression was observed at the initial diagnosis (I; 84.3%), and that the expression then diminished after treatment with chemotherapy with rituximab (II; 7.1%). Interestingly, CD20 protein expression was observed again at the terminal stage after several chemotherapy treatments without rituximab (III; 76.4%). On the other hand, CD19 expression level was stable throughout the clinical course.

Figure 3. Alteration of CD20 mRNA expression in B-cell lymphoma cells during the clinical course. (A) RT-PCR (RT) was performed using total RNA from the same tumor cells as in Figure 2B (UPN #3 in Table 2). As positive and negative controls, total RNA from Raji and 293T cells was utilized (lanes 1 and 2), respectively. I, II, and III (lanes 3 to 5) correspond to the clinical stages depicted in Figure 2A. (B) Quantitative RT-PCR was performed using the same RNA as in (A). Arbitrary units of CD20 mRNA
expression are indicated in the vertical axis. Note that faint expression of CD20 mRNA could be seen at stage II (column 4), despite a loss of CD20 surface protein expression as shown in Figure 2B. (C) CD20 mRNA expression in the lymphoma cells of UPN #4 (Table 2) was also analyzed. Tumor cells were derived from cerebral fluid at the first relapse after chemotherapy without rituximab (lane 1). Although complete remission was obtained after using rituximab-containing salvage chemotherapy, a second relapse occurred. Tumor cells were once again harvested from this patient’s cerebral fluid, and analyzed (lane 2). (D) Quantitative RT-PCR was also performed using the same RNA as in (C). Note that CD20 mRNA expression was significantly diminished but could still be observed. In these cells, CD20 protein expression was undetectable using FCM or IHC as indicated in Table 2. Positive and negative controls derived from Raji and 293T cells are indicated as (+) and (-).

Figure 4. Restoration of CD20 mRNA and protein expression by treatment with the DNA methyltransferase inhibitor 5-aza-2’-deoxycytidine (5-Aza).

(A) Primary B-cell lymphoma cells, which showed a CD20 protein-negative phenotype from UPN #3, were incubated with or without 5-Aza. Total RNA was prepared, and semi-quantitative RT-PCR was performed. Restoration of CD20 mRNA expression after treatment with 5-Aza was observed in lane 3. As a positive control, tumor cells obtained at the initial diagnosis of that same patient were utilized in lane 1. (B) The CD20 protein-negative B-cell lymphoma cell line RRBL1, which was derived from UPN #5 in Table 2, was incubated in culture medium with or without 5-Aza. After preparation of total RNA and whole-cell lysates from these cells, semi-quantitative RT-PCR and immunoblotting (IB) were performed. Up-regulation of CD20 mRNA and protein expression was observed as shown in lane 2. (C) Quantitative RT-PCR was performed using the same mRNA as in (B). We observed an up-regulation of more than 10-fold in CD20 mRNA after treatment with 5-Aza (column 2). (D) RRBL1 cells were treated with 5-Aza under the same conditions as in (B), and FCM analysis using anti-CD20 antibody was performed. After treatment with 5-Aza, 30.7% of RRBL1 cells showed a CD20 positive phenotype. Positive cells are shown with black lines, and the percentage of positive cells is also shown. (E) In vitro ADCC analysis using the ⁵¹Cr-release assay. Cells from the CD20-positive B-cell lymphoma/leukemia cell lines Daudi and RRBL1
treated with or without 5-Aza were used for this assay. In Daudi cells (white circles), but not in RRBL1 cells (white squares), cytotoxic activity was observed in the presence of rituximab in a dose-dependent manner. Partial restoration of rituximab sensitivity in RRBL1 cells was observed after treatment with 5-Aza (black triangles). Error bars indicate ± 1 standard deviation.
Table 1. CD20-positive B-cell malignancies treated with rituximab in Nagoya University Hospital

<table>
<thead>
<tr>
<th>Disease Status</th>
<th>No. of patients</th>
<th>Disease status at rituximab therapy</th>
<th>Response</th>
<th>Re-sampling of tumor tissue</th>
<th>CD20 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st</td>
<td>RD/PD</td>
<td>1st</td>
<td>RD/PD</td>
</tr>
<tr>
<td>DLBCL</td>
<td>51</td>
<td>45</td>
<td>6</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>FL</td>
<td>43</td>
<td>26</td>
<td>17</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Nodal marginal zone BCL or MALT</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Burkitt or Burkitt like</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Mediastinal large B-cell</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Intravascular large B-cell</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mantle cell</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lymphoplasmacytic</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CLL/SLL</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total cases</strong></td>
<td><strong>124</strong></td>
<td><strong>96</strong></td>
<td><strong>28</strong></td>
<td><strong>36</strong></td>
<td><strong>19</strong></td>
</tr>
</tbody>
</table>

DLBCL; diffuse large B-cell lymphoma, FL; follicular lymphoma, BCL; B-cell lymphoma, MALT; mucosa-associated lymphoid tissue, CLL/SLL; clonic lymphocytic leukemia / small lymphocytic lymphoma, 1st; the first treatment, RD/PD; relapse or progression, +/-; positive / decreased / negative.
**Table 2. CD20-negative RD/PD after treatment with rituximab-containing combination chemotherapy**

<table>
<thead>
<tr>
<th>UPN</th>
<th>age/sex</th>
<th>diagnosis (admission)</th>
<th>status</th>
<th>chemo. regimen</th>
<th>total ritux.</th>
<th>duration until CD20(-)</th>
<th>diagnosis (RD/PD)</th>
<th>patho. source</th>
<th>FCM</th>
<th>IHC</th>
<th>RT</th>
<th>CDS mutation</th>
<th>survival after CD20(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65 / M</td>
<td>DLBCL</td>
<td>2 rel.</td>
<td>R-salvage</td>
<td>8</td>
<td>2M</td>
<td>DLBCL</td>
<td>BM</td>
<td>(-)</td>
<td>(-)</td>
<td>N.A.</td>
<td>S97F [2/16]</td>
<td>6M †</td>
</tr>
<tr>
<td>2</td>
<td>37 / F</td>
<td>DLBCL</td>
<td>1 diag.</td>
<td>R-CHOP</td>
<td>8</td>
<td>9M</td>
<td>DLBCL</td>
<td>BM</td>
<td>(±) b)</td>
<td>(±) c)</td>
<td>N.A.</td>
<td>V247I [2/10]</td>
<td>4M †</td>
</tr>
<tr>
<td>3</td>
<td>75 / M</td>
<td>DLBCL</td>
<td>1 diag.</td>
<td>R-CHOP</td>
<td>7</td>
<td>10M</td>
<td>DLBCL</td>
<td>BM,CF</td>
<td>(-)</td>
<td>(-)</td>
<td>↓</td>
<td>WT</td>
<td>11M †</td>
</tr>
<tr>
<td>4</td>
<td>42 / M</td>
<td>FL G1</td>
<td>2 rel.</td>
<td>R-CHOP</td>
<td>4</td>
<td>23M</td>
<td>DLBCL</td>
<td>BM</td>
<td>(-)</td>
<td>(-)</td>
<td>↓</td>
<td>WT</td>
<td>11M †</td>
</tr>
<tr>
<td>5</td>
<td>52 / M</td>
<td>FL G2</td>
<td>3 rel.</td>
<td>R-cMOPP</td>
<td>14</td>
<td>81M</td>
<td>DLBCL</td>
<td>BM,LN a)</td>
<td>(-)</td>
<td>(-)</td>
<td>↓</td>
<td>WT</td>
<td>8M †</td>
</tr>
</tbody>
</table>

a) Lymphnode sample obtained at his autopsy, b) 19% of tumor cells were CD20 positive, c) 30% of tumor cells showed CD20 positive. Status; disease status of those patients at the first treatment with rituximab, rel.; relapse, duration until CD20(-); duration (months) until CD20(-) relapse or PD from the first rituximab treatment, total ritux.; total times of rituximab treatment, patho. source; sources of tumor tissues for pathological analysis, BM; bone marrow, CF; cerebral fluid, LN; lymphnode, RT; RT-PCR, N.A.; not available, black arrow; down-regulated, CDS; coding sequences, survival after CD20(-); duration from CD20-negative change until death.
Table 3. Response against salvage chemotherapies for the 36 patients who showed relapse or PD after rituximab-containing chemotherapies.

<table>
<thead>
<tr>
<th>response</th>
<th>CD20 expression</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(-)</td>
<td>(+)</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>0</td>
<td>5</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD/PD</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>death</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>5</td>
<td>14</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[cases]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CR; complete remission, PR; partial response, SD; stable disease, RD/PD; relapse/progression. These outcomes were evaluated in July, 2007.
Figure 1AB
Figure 1C

**C**

**UPN #3**

<table>
<thead>
<tr>
<th>HE (CS)</th>
<th>HE (CS)</th>
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</thead>
<tbody>
<tr>
<td>CD20 (CS)</td>
<td>CD20 (CS)</td>
</tr>
<tr>
<td>CD10 (CS)</td>
<td>CD10 (CS)</td>
</tr>
</tbody>
</table>

**diagnosis** | **RD/PD**

For personal use only.
A  UPN #3

Rituximab (+)

0 12 24 [Months]

I II III

B

CD20  CD19

I

84.3  96.8

II

7.1   97.4

III

76.4  89.5

Figure 2
Figure 3

A  **UPN #3**

<table>
<thead>
<tr>
<th></th>
<th>(+)</th>
<th>(-)</th>
<th>I</th>
<th>II</th>
<th>III</th>
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<tbody>
<tr>
<td>1</td>
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<td></td>
<td></td>
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<tr>
<td>2</td>
<td></td>
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</tbody>
</table>

RT

- **CD20**
- **β-actin**

B  **qRT-PCR**

CD20

Arbitrary units

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>(+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)</td>
<td></td>
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C  **UPN #4**

<table>
<thead>
<tr>
<th></th>
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<tbody>
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</tr>
<tr>
<td>2</td>
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</table>

RT

- **CD20**
- **β-actin**

D  **qRT-PCR**

CD20

Arbitrary units

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>(+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)</td>
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</tbody>
</table>

For personal use only.
Figure 4

A  **UPN #3**

<table>
<thead>
<tr>
<th>5-Aza</th>
<th>-</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
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<td></td>
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</tr>
<tr>
<td>RT</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

B  **RRBL1 cells**

<table>
<thead>
<tr>
<th>5-Aza</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRBL1</td>
<td></td>
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<tr>
<td>CD20</td>
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</tr>
<tr>
<td>β-actin</td>
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<td></td>
</tr>
<tr>
<td>RT</td>
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<td>2</td>
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</table>

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD20</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td></td>
</tr>
</tbody>
</table>

C  **Graph**

 Arbitrary units

1  1000  100  10  1

1  2
Figure 4

**D**

5-Aza (-)

5-Aza (+)

0.22

30.7

**E**

ADCC activity

- Daudi
- RRBL1
- RRBL1+5-Aza

[μg/ml]

Figure 4
Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance

Junji Hiraga, Akihiro Tomita, Takumi Sugimoto, Kazuyuki Shimada, Masafumi Ito, Shigeo Nakamura, Hitoshi Kiyoi, Tomohiro Kinoshita and Tomoki Naoe