Identification of CD20 C-Terminal Deletion Mutations Associated with Loss of CD20 Expression in Non-Hodgkin’s Lymphoma

Yasuhiro Terui,1,3,4 Yuji Mishima,3,4 Natsumiko Sugimura,4 Kiyotsugu Kojima,4 Takuma Sakurai,5 Yoko Mishima,1,3 Ryoko Kuniyoshi,3,4 Akiko Taniyama,3,4 Masahiro Yokoyama,1 Sakura Sakaji,1,3 Kengo Takeuchi,2 Chie Watanabe,1 Junji Takahashi,1,3 Yoshinori Ito,1,3 and Kiyohiko Hatake1,3,4

Abstract

Purpose: Rituximab is commonly incorporated into CD20-positive B-cell lymphoma therapy to improve response and prognosis. With increasing use, resistance to rituximab is a continuing concern, but CD20 mutation as a cause of resistance has not previously been reported.

Experimental Design: Freshly collected lymphoma cells from 50 patients with previously untreated or relapsed/resistant non-Hodgkin’s B-cell lymphomas (diffuse large B cell, n = 22; follicular, n = 7; mucosa associated lymphoid tissue, n = 16; chronic lymphocytic leukemia, n = 2; small lymphocytic lymphoma, n = 1; lymphoplasmacytic, n = 1; mantle cell lymphoma, n = 1) were assessed for CD20 expression by flow cytometry, and CD20 gene sequencing was done on extracted DNA.

Results: CD20 mutations were found in 11 (22.0%) of 50 patients and could be grouped as C-terminal deletion (8.0%), early termination (10.0%), and extracellular domain (2.0%) mutations. The mean fluorescence intensity of CD20 on fresh lymphoma cells was significantly lower for the C-terminal deletion mutation [3.26; 95% confidence interval (95% CI), 0.09-6.89] compared with wild type (30.8; 95% CI, 22.4-39.2; P < 0.05). In contrast, early termination mutations did not show significant differences in CD20 expression compared with wild type (19.5; 95% CI, 10.7-28.4; P > 0.05).

Conclusions: It is possible that C-terminal deletion mutations of CD20 may be related to relapse/resistance after rituximab therapy. These mutations should be examined in patients showing progression of disease after partial remission.

Therapeutic monoclonal antibodies have been developed against cancer cells, such as malignant lymphoma, breast, and colorectal cancers, including rituximab (Mabthera/Rituxan; ref. 1), trastuzumab (Herceptin; ref. 2), and bevacizumab (Avastin; ref. 3), respectively. The rituximab target antigen is the B-cell membrane differentiation antigen CD20, and rituximab has emerged as a useful tool for adjunct cancer therapy (4). Although CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone/prednisolone) therapy leads to median overall survival rates of only 60%, addition of rituximab improves rates by ~20% (5).

With the need to determine standard first-, second-, and subsequent-line combination therapies using rituximab (6, 7), relapse/resistance to rituximab therapy is an important issue. The mechanisms of action of rituximab are inhibition of proliferation, induction of apoptosis, complement-dependent cytotoxicity, and antibody-dependent cellular cytotoxicity. A few reports indicate that loss of CD20 expression occurs in some patients with non-Hodgkin’s lymphoma during rituximab therapy (8–10), but the relationship between development of resistance to rituximab and changes in rituximab action have not yet been clarified. Heterogeneity of intensity of CD20 expression in replicate analysis of the same sample has been commonly observed by flow cytometric analysis (11). One explanation for this might be the development of resistant subsets of lymphoma cells by mutation. Recently, mutations in the epidermal growth factor receptor have been reported to have a relationship with the differing sensitivity to gefitinib therapy seen in samples from Japanese and American patients (12).

Our experience with resistance began with a patient who had a posterior mediastinal lymphoma that became resistant during...
rituximab plus CHOP therapy. Initially, pathologic examination by computed tomography–guided biopsy and immunohistologic testing showed that the lymphoma cells expressed the CD20 antigen. During rituximab plus CHOP therapy, the patient experienced a massive right pleural effusion with refractory disease. Second, if the patients have lymphoma cells with this kind of the mutation, it will be possible that they may be treated with other strategies such as other anti-CD20 antibodies with or without radioisotopes and anti-CD22 antibodies with or without calicheamicin. For those reasons, this work will be applied to future important practice of the field of malignant lymphoma.

**Materials and Methods**

**Collection of clinical samples.** This study was approved by the ethics committee of the chamber of physicians at the Japanese Foundation for Cancer Research, Japan. Written informed consent was obtained from all patients to use the resected samples and to be bone marrow aspirates for research purposes. For this study, all 50 patients with malignant lymphoma who underwent lymph node biopsy and bone marrow aspiration at the Cancer Institute Hospital of the Japanese Foundation for Cancer Research between February 1, 2003, and November 30, 2004, were assessed prospectively. After histopathologic examination, the malignancies were classified according to WHO lymphoma criteria. Forty-three patients received R-CHOP (rituximab 375 mg/m² weekly for 8 cycles and cyclophosphamide 750 mg/m², doxorubicin 50 mg/m², vincristine 1.4 mg/m², and prednisolone 60 mg/m²) therapy. Three of them underwent radiotherapy to a total dose of 35 to 45 Gy after R-CHOP, and one of them received radiation therapy before R-CHOP. One patient received a therapy of rituximab 375 mg/m² weekly for 8 cycles and cyclophosphamide 750 mg/m², vincristine 1.4 mg/m², and prednisolone 60 mg/m². Three of them underwent radiotherapy to a total dose of 35 to 45 Gy after R-CHOP, and one of them received radiation therapy before R-CHOP. One patient received a therapy of rituximab 375 mg/m² weekly for 8 cycles and cyclophosphamide 750 mg/m², vincristine 1.4 mg/m², and prednisolone 60 mg/m². Five patients received rituximab monotherapy (375 mg/m² weekly for 8 cycles). For one patient, rituximab-VP-16 was given as rituximab 375 mg/m² weekly for 8 cycles, and etoposide 50 mg was administered orally for 2 of every 4 wks.

Fresh lymphoma cells were collected from 50 patients with non-Hodgkin’s lymphoma (diffuse large B cell, n = 22; follicular, n = 7; mucosa associated lymphoid tissue, n = 16; chronic lymphocytic leukemia, n = 2; small lymphocytic lymphoma, n = 1; lymphoplasmacytic, n = 1; mantle cell lymphoma, n = 1). In 9 of the 50 patients, analysis of the CD20 gene was done after disease progression.

**Surface markers.** The CD19-positive cells isolated by a magnetic cell sorting system were stained with phycoerythrin-conjugated anti-CD19 (BD Biosciences) and phycoerythrin-conjugated anti-CD20 antibodies. Flow cytometry was done by FACScan (Becton Dickinson). Intensity of CD20 expression was normalized by comparison against a control and expressed as the mean fluorescence intensity ratio. Rituximab was labeled with Alexa Fluor 488 molecule (Invitrogen) in accordance with the manufacturer’s instructions.

**Assessment of mutations and expression.** Genomic DNA and total RNA were extracted from CD19-positive lymphoma cells in TRIzol reagent (Invitrogen) using the supplied protocol. One microgram of RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (BD Biosciences) using oligo(dT)₁₇ according to the manufacturer’s instruction. Genomic PCR of five of the eight exons of the CD20 gene was done using BD Advantage 2 polymerase. Reverse transcription-PCR (RT-PCR) was also done using the following pairs of primers containing BamHI and SalI sites to amplify the full-length transcript and selected exon pairs: exons 3 and 4, 5, and 6, and 7 and 8. PCR amplification was carried out with the Hot Start/Amplimix method with the following temperature cycling parameters: 95°C for 30 s, 58°C for 30 s, 72°C for 30 s for 25 cycles, and a final extension at 72°C for 3 mins. The primer pair sequences used for amplification are available as below.

Genomic PCR was done using the following primers with five of eight exons of the CD20 gene, respectively: Forward primer for exon 3, 5'-CCTTCTCTAGACATGTGGGA-3', reverse primer for exon 3, 5'-ACTGACATTACCCCCCAAGCTTGAGTCCC-3'; forward primer for exon 4, 5'-CTCTCCCCGCCTCTCAGATTAGTG-3'; reverse primer for exon 4, 5'-TTTTCCTACCCCATATGCGTCCCCAGAGAGAG-3'; forward primer for exon 5, 5'-CTCTCTATCTGTCGTCCTGCCC-3'; reverse primer for exon 5, 5'-AAAAATTAGGTACTCTCTG-3'; reverse primer for exon 6, 5'-CTTTCCATTGTTGGGA-3'; forward primer for exon 6, 5'-CATTTCCAGTCAAAGAAAAATGAT-3'; reverse primer for exon 6, 5'-ACTTACCAAGACATCTACACAAAGA-3'; forward primer for exon 7, 5'-TGTTTTCTCAGGACATTGTTGAGCTTCTA-3'; reverse primer for exon 7, 5'-ACTGACATTACAGAGTGTTGAGGAGGCA-3'; forward primer for exon 8, 5'-TTTGCAGTTTAGAATAGTGCCTCCTGTA-3'; and reverse primer for exon 8, 5'-CAGAAAAACGAAGAATACACTITAGGAGAG-3'.

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**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Histology</th>
<th>Treatment</th>
<th>n</th>
<th>Analysis of PD sample</th>
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<td>MALT</td>
<td>R-CHOP</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R-VP16</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>FL</td>
<td>R-CHOP</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R-CHOP -- RTx</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DLBCL</td>
<td>R-CHOP</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R-CHOP -- RTx</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CLL/ SLL</td>
<td>R-CHOP</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RTx -- R-CHOP</td>
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<td>R-COP</td>
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<td>1</td>
</tr>
<tr>
<td>MCL</td>
<td>CHOP+ RTx</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: CLL/SLL, chronic lymphocytic leukemia or small lymphocytic lymphoma; COP, cyclophosphamide, vincristine, and prednisone; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MALT, mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; PD, progressive disease; R, rituximab; RTx, radiation therapy; VP16, etoposide.
RT-PCR was also done using the following pairs of primers with *Bam*HI and *Sal*I sites for full length and three parts of exons (exons 3 and 4, 5 and 6, and 7 and 8, respectively: Forward primer for full length, 5'-CGGGATCCCGGATGACAACACCCCCAGA-3'; reverse primer for full length, 5'-TCCCCCGGGGATTAAAGGAGATTGTC-3'; forward primer for exons 3 and 4, 5'-ATGACAAACCCAGAAATTCAGTAAATGGG-3'; reverse primer for exons 3 and 4, 5'-CATAATGCTCCAGAAGGGG-TACCACAC-3'; forward primer for exons 5 and 6, 5'-TATAATTTCCCG-GATCACTTCTCCAGCA-3'; reverse primer for exons 5 and 6, 5'-CAGAGGCAGCCTGATGCTAAACGTAAC-3'; forward primer for exons 7 and 8, 5'-GATAATTGTCCGTAGCTGATGTTCCT-3'; and reverse primer for exons 7 and 8, 5'-TTAAGGAGAGCTCCTATTGCTTGTAGA-3'. The following pair of primers for gyceraldehyde-3-phosphate dehydrogenase was used as a housekeeping gene control: forward primer, 5'-CCTTTTGGCTCACTTCCAGAGAGGG-3'; and reverse primer, 5'-AGTATGCTTGCTGACCTGCTTGTG-3'.

Direct sequence analysis of genomic DNA and PCR product was done by ABI PRISM 3100 (Invtrogen) as described in previous reports (13, 14).

**Cloning and expression of CD20 mutations.** The PCR product was subcloned into mammalian expression vector pTARGET (Promega) using a single 3-T overhang into the cloning site. pTARGET vectors with CD20 mutants were stably introduced into the chronic myelogenous leukemia cell line K562 by electroporation and selected with G418 (Invitrogen).

**In vitro translation assay.** The CD20 mutant genes in the pTARGET vector were transcribed and translated using an *in vitro* translation kit (Promega) according to the protocol. In brief, 1 µg of DNA was added to the *in vitro* translation reaction mixture. After adding 1 Ci of 35S-methionine, the reaction mixture was incubated at 30°C for 1 h.

After electrophoresis of the labeled products, the gel was dried and autoradiography was done.

In **vivo transfection assay.** Wild-type and mutant sequences were stably transfected into K562 cells (K562/mock, K562/WT, K562/CD-1, K562/CD-2, and K562/CD-3); after which, flow cytometric analysis was done and confocal laser scanning microscopy was used for imaging analysis (FV1000, Olympus). Immunohistochemistry was done on mock, wild-type, CD-1, CD-2, and CD-3 mutant–transfected K562 cells using a biotin-free dextran polymer system (Envision+, DAKO).

**Antibody against CD20 N-terminal peptide.** The peptide corresponding to amino acids 23 to 36 of CD20, MQSGPKPLFRRMSS, was synthesized, and a polyclonal antibody against the peptide was raised in the rabbit by Scrum, Inc. The antiserum was purified using an Immunopure IgG purification kit (Pierce Biotechnology, Inc.) according to the manufacturer's protocol. For Western blot analysis with this antibody, the cells were then washed once with PBS and lysed with 1× sample buffer. After electrophoresis on 10% to 20% gradient gels (Daichikagaku), Western blot analysis was done with primary polyclonal anti–CD20 N-terminus antibody (dilution, 1:1,000) and secondary horse radish peroxidase–conjugated anti–rabbit immunoglobulin antibody (Santa Cruz Biotechnology, Inc.). Detection was done using an enhanced chemiluminescence system (GE Healthcare UK Ltd.).

**Immunohistochemistry.** Sections (4-µm thick) were cut and mounted on poly-L-lysine–coated slides. Immunohistochemistry was done using a biotin-free dextran polymer system (Envision+, DAKO). Briefly, after deparaffinization in xylene and rehydration using ethanol/water dilutions, antigen retrieval was done by placing the sections in preheated 0.01 mol/L citrate buffer (pH 6) for 40 mins at 97°C, followed by 20 mins at room temperature. Endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide for 5 mins at room temperature. The sections were incubated with the anti-N-terminus of CD20 rabbit polyclonal antibody (1:400) for 30 mins at room temperature. The antibody was detected with Envision+, and the reactions were visualized by incubating the sections with diaminobenzidine (DAB+, DAKO). The sections were counterstained with hematoxylin; all staining procedures were done in a DAKO Autostainer.

![Fig. 1. CD20 expression and mutation analysis in CD19-positive lymphoma cells from a patient with posterior mediastinal lymphoma. A, flow cytometry for CD19 and CD20 cells. B, RNA analysis. RT-PCR using CD20 primers. Total RNAs from Raji and K562 cells were used as positive and negative controls, respectively. C, nucleic acid sequence analysis. Arrows, replacement or insertion of a nucleic acid. Top and bottom, sequences of nucleic acids and amino acids of wild-type and mutant (PT-1 and PT-2) sequences corresponding to amino acids 214 to 240 and 231 to 241. WT, wild type; a.a., amino acids. D, amino acid sequence analysis. Two clones (PT-1 and PT-2) showed replacement of one amino acid and a partial deletion in the C-terminal cytoplasmic domain. Four amino acids (E237-Y240) were also changed at the C-terminus (R237-Y240). PT, patient.](https://www.aacrjournals.org/doi/fig/1)
Clinical parameters. Time to progression was calculated from the date of initiation of rituximab therapy to the date of detection of progressive disease or to the date of last contact.

Statistical analysis. Statistical analysis was done using StatView version 5.0 and InStat version 2.00 software (SAS Institute, Inc.). Statistical comparisons were done by Kruskal-Wallis nonparametric ANOVA test and confirmed by Student’s t test, with \( P < 0.05 \) interpreted as a significant difference. Time to progression was analyzed by the Kaplan-Meier method using Dr. SPSS II software (SPSS Japan, Inc.), and the log-rank test was used for univariate analysis.

Results

CD20 mutations. Patient characteristics and timing of mutation analysis are shown in Table 1. The index case of a female with posterior mediastinal lymphoma developing resistance during rituximab plus CHOP therapy was included in this nucleic acid analysis. Although these lymphoma cells were CD19 positive and CD20 negative on flow cytometric analysis (Fig. 1A), CD20 mRNA was detectable by RT-PCR (Fig. 1B). In our study, genomic PCR and RT-PCR were done with the primers for five of the eight exons in the CD20 genes.

In exon 8, sequence analysis for genomic DNA and the PCR product revealed that there were some clones with frameshifts due to insertion of one adenine residue. To confirm this result, the PCR products were subcloned into mammalian expression vector pTARGET, and analysis on the ABI sequencer detected the same frameshift mutation in combination with two different point mutations (Fig. 1C). Four of the 10 clones identified showed the same frameshift mutation in genomic DNA that had been detected by PCR. Of the two point mutations, both resulted in replacement of one amino acid (T219A and E215G) and both were seen in combination with a partial deletion frameshift mutation in the C-terminal cytoplasmic domain (changing the C-terminal four amino acids from EQTI to RTDY; Fig. 1D).

RNA samples from 49 other patients with non-Hodgkin’s lymphoma were investigated retrospectively by RT-PCR analysis.

Table 2. Mutations found in 11 patients

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mutated domains</th>
<th>Amino acid sequence from mutation</th>
<th>Classification</th>
<th>Therapy</th>
<th>Biopsy after PD</th>
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<tbody>
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<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-terminal deletion</td>
<td>C-terminal cytoplasmic</td>
<td>I211S</td>
<td>FL</td>
<td>R-CHOP</td>
<td>Yes</td>
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<tr>
<td>(truncation) CD-1</td>
<td>C-terminal cytoplasmic</td>
<td>EQT123 RTDY</td>
<td>DLBCL</td>
<td>R-CHOP</td>
<td>No</td>
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<tr>
<td>CD-2</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD-3*</td>
<td>C-terminal cytoplasmic</td>
<td>T219A: EQT123 RTDY</td>
<td>DLBCL*</td>
<td>RTx → R-CHOP</td>
<td>Yes*</td>
</tr>
<tr>
<td>CD-4*</td>
<td>C-terminal cytoplasmic</td>
<td>E215G: EQT123 RTDY</td>
<td>DLBCL*</td>
<td>RTx → R-CHOP</td>
<td>Yes*</td>
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<tr>
<td>CD-5</td>
<td>Second transmembrane fused to C-terminal cytoplasmic</td>
<td>SLLAATEKNSRKCLVKGMIMNSLFLAAAITS- GMILSIMDL fused to ITPGSNGEKLOEV-FGQKNDNFEFIEPLCCHFWDNSFNHGHT</td>
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<td>CHOP + RTx → R</td>
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<td>N-terminal cytoplasmic</td>
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<td>DLBCL</td>
<td>R-CHOP</td>
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</table>

*Clones CD-3 and CD-4 are from the same patient.

Fig. 2. Relationship between mutations and CD20 expression. Mean fluorescence intensity of CD20 relative to the control was assessed in CD19-positive cells from clinical samples by flow cytometric analysis. Fifty cases were classified as nonmutations or as mutations and grouped according to the domain affected (C-terminal deletion, extracellular domain, early termination, and transmembrane domain). MFI, mean fluorescence intensity.
All patients had received rituximab with or without other chemotherapy or radiotherapy, and in 9 of the 50 patients, fresh samples for the analysis were taken after disease progression (Table 1). We found heterogeneity at the nucleic acid level, with several different CD20 mutation types identified by DNA analysis, which could be grouped according to their location (Table 2). The C-terminal cytoplasmic domain was affected in patients classified in Group 1. Table 2 presents the group 1 mutations seen in the index case (CD-3, CD-4), in which the adenine insertion frameshift was observed without detection of the additional point mutation (CD-2) and a partial deletion stopped at amino acid S211 (CD-1). Finally, a replacement of ITPGSNGEKLQEVFGQRKNDNEFIEPLCC-HFWNSFHIGHT at S162 in the second transmembrane domain caused the C-terminal cytoplasmic domain to be defective (CD-5). The samples from three of the four patients in group 1 were taken after disease progression.

In group 2, the extracellular domain was altered by replacement of an amino acid (T180A). In group 3, replacement of an amino acid (F125L) altered the third transmembrane domain. The four patients in group 4 had a stop codon detected close to the 5' site of the CD20 gene, which may produce a short peptide. In these cases, a second methionine following the stop codon may initiate transcription of a long peptide.

**Relationship between CD20 expression and CD20 mutations.** The relationship between groups of mutations and CD20 expression were examined in fresh CD19-positive cells from patients with non-Hodgkin's lymphoma. To observe which group of CD20 mutations was related to down-regulation of CD20 expression, the mean fluorescence intensity of CD20 expression relative to the control was examined in each group (Fig. 2). There was a significant difference in CD20 expression between wild-type and C-terminal deletion mutation groups (mean difference, 24.0; *P* < 0.01), but this was not the case for wild type compared with early termination groups (mean difference, 3.1; *P* > 0.05) or between C-terminal deletions mutation and early termination groups (mean difference, -21.0; *P* > 0.05). The CD20 expression seen in group 1 [mean fluorescence intensity, 3.26; 95% confidence interval (95% CI), 0.09-6.89] significantly decreased compared with wild type.
(mean fluorescence intensity, 30.8; 95% CI, 22.4-39.2; \( P < 0.05 \); two-sided Student’s \( t \) test), whereas that of the early termination group (mean fluorescence intensity, 19.5; 95% CI, 10.7-28.4) was not significantly different from wild type. In addition, there was no significant difference in the mean fluorescence intensity among between the different subtypes of B-cell lymphomas such as diffuse large B-cell lymphoma (mean fluorescence intensity, 35.9; 95% CI, 23.5-48.3), mucosa-associated lymphoid tissue (mean fluorescence intensity, 32.8; 95% CI, 18.3-47.3), follicular lymphoma (mean fluorescence intensity, 17.9; 95% CI, 11.7-24.1), and chronic lymphocytic leukemia or small lymphocytic lymphoma (mean fluorescence intensity, 18.5; 95% CI, 7.3-29.7) did not significantly show low expression of CD20 as compared with those at diagnosis (mean fluorescence intensity, 18.5; 95% CI, 7.3-29.7). These results suggest that the C-terminal deletion mutation is strongly associated with decreased or absent CD20 expression. One of the reported mechanisms of action for rituximab is complement-dependent cytotoxicity, which is regulated by some inhibitory factors such as CD46, CD59, and CD55 (15, 16). Because CD55 is a potent inhibitor of rituximab-induced complement-dependent cytotoxicity in bulky lymphomas (17), CD55 expression was examined in the fresh CD19-positive cells from the patients, but no significant difference was detected for CD55 expression on the lymphoma cells (data not shown).

**CD20 production in vitro and in vivo.** The in vitro translation and in vivo transfection experiments done to examine CD20 production showed that cells with C-terminal deletion mutations (CD-2, CD-3, and CD-4) had lower levels of RNA and protein than cells that were wild type or contained other point mutations (data not shown). To confirm whether C-terminal deletion mutations reduce or eliminate CD20 expression on the cell surface, the mutated genes subcloned into pTARGET were stably transfected into K562 cells (Fig. 3). K562/mock and K562 cells did not express CD20 molecules on flow cytometric (Fig. 3A) and microscopic (Fig. 3B) analyses. CD20 expression on K562/CD-1, K562/CD-2, and K562/CD-3 cells was not detected or showed a very low signal on flow cytometric (Fig. 3A) and microscopic (Fig. 3B) analyses. These results were not due to a loss or decrease in CD20 RNA as examined by RT-PCR (Fig. 3C). Mutant products CD-2 and CD-3 were expressed in addition to wild type, although fewer larger size fragments were deleted than that of wild type (Fig. 3D). On immunostaining with anti-N-terminal CD20 antibody, wild-type product was strongly detected on the cell membrane (Fig. 3E); C-terminal deletion mutants were weakly detected in the cytoplasm but not on the cell surface.

**Discussion**

The results from the original index case suggested that replacement of one amino acid and/or the partial deletion of the C-terminus might cause loss of CD20 expression, and hence, analysis was expanded retrospectively to include 50 patients. In these 50 patients, the overall response rate was 92% (46 of 50) after rituximab therapy, but two of these patients developed progressive disease after achieving a partial response. In fact, two of the three patients with mutations detected after disease progression (Table 2) showed C-terminal deletions. Because C-terminal deletion mutations are associated with reduced or absent expression of CD20, we investigated whether there was any significant difference in response and prognosis for patients after rituximab therapy between this group and the wild-type group. Complete response rates with rituximab therapy were 49% in the wild-type group but only 25% in the C-terminal deletion mutation group. No statistically significant difference between these groups was found because of the low number of cases in the C-terminal deletion mutation group. After rituximab therapy, median time to progression was 31 months (95% CI, 18-44 months), 30 months (95% CI, 31-37 months), and 7 months (95% CI, 0-18 months) for the wild-type, early-termination, and C-terminal deletion groups, respectively.

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**Fig. 4.** The structures of wild-type and mutant CD20. Wild-type CD20 is composed of 297 amino acids (A), and casein kinase 2 and calcium/calmodulin-dependent protein kinase 2 phosphorylation sites are expected in its C-terminal cytoplasmic domain. C-terminal deletion mutants are shown as CD-1 (B), CD-2 (C), CD-3 (D), and CD-4 (E).
Although the tumor types and the treatment received were heterogeneous and only four patients had C-terminus mutations, the C-terminal deletion mutation seems to be associated with short time to progression and early relapse of disease.

The mean fluorescence intensity results indicate that the C-terminal deletion mutation is strongly associated with decline or disappearance of CD20 expression, and the results of expression studies suggest that C-terminal deletions may mask CD20 expression on the cell surface or affect duration of cell surface exposure to CD20.

Heterogeneity of intensity of CD20 expression in replicate analysis of the same sample is commonly observed with flow cytometric analysis (11). This indicates that subclones expressing lower CD20 levels are present in CD20-positive lymphoma cells and that surviving clones may cause resistance or relapse after rituximab therapy. It is thus vital that these clones are killed to protect patients from the risk of resistance or relapse. Jazirehi et al. (18) have reported that rituximab-resistant lymphoma cells can be chemosensitized following treatment with pharmacologic inhibitors such as bortezomib that target survival/antiapoptotic pathways. Structurally, the C-terminal cytoplasmic domain of CD20 possesses some phosphorylation sites for protein kinases such as casein kinase 2 and calcium/calcmodulin-dependent protein kinase 2 (Fig. 4A). S239 is predicted to be phosphorylated by casein kinase 2, and S221 and S225 are potential calcium/calmodulin-dependent protein kinase 2 phosphorylation sites (19, 20); however, the significance of the phosphorylation of these sites remains to be clarified. On the other hand, the cytoplasmic region of CD20 (amino acids 219–225) is known to be required for its redistribution to the detergent-insoluble membrane compartment, which plays an important role in the action of rituximab (21). One of four C-terminal deletion mutants (Fig. 4B) reported here had lost several predicted phosphorylation sites such as casein kinase 2 and calcium/calmodulin-dependent protein kinase 2 in contrast to the other three mutants (Fig. 4C-E). Another feature of the distal region in the C-terminus is the presence of a glutamic acid–rich region (19, 22). The sequence of E233 to E292 is predicted to be a glutamic acid–rich region profile using the MotifScan program and PROSITE database, and this region may play an important role in retention of calcium ions, analogous to the role of bone sialoprotein (23). It has been reported that B lymphocytes are activated and CD20 is up-regulated by phorbol myristate acetate and ionomycin (24), suggesting that intracellular calcium ions participate in CD20 expression. However, we have shown that the C-terminal deletion mutant CD20 was produced as RNA in the cells but was not detected as a protein on the cell surface. This may be a consequence of the rapid turnover of CD20 mutant molecules between the cell surface and cytoplasm, resulting in exposure at the cell surface that is too brief for detection by immunofluorescence. If so, anti-CD20 antibody linked to anticancer drugs such as ozogamicin could be a useful treatment approach for patients with this mutation.

Two classes of mutations are spontaneous mutations and induced mutations caused by mutagens (25, 26). Spontaneous mutations on the molecular level include tautomerism, depurination, deamination, transition, and transversion, whereas chemicals such as alkylating agents and radiation can induce mutations on the molecular level. Alkylating agents such as cyclophosphamide in CHOP therapy can mutate replicating and nonreplicating DNA and has certain effects that then lead to transitions, transversions, or deletions. In this study, 44 patients had received CHOP therapy with rituximab, and three of them (6.9%) had C-terminal deletion mutants when they showed progression disease after R-CHOP therapy. One patient showed C-terminal deletion before R-CHOP therapy. Because Ragg et al. (27) has reported that overexpression and mutant of methylguanine methyltransferase protects mice against effect of alkylators, loss of function of this enzyme may induce gene mutagenesis by alkylating reagents such as cyclophosphamide. Moreover, 4 of 50 cases received radiation therapy during the treatment, and radiation therapy before administration of rituximab was given to two cases, which showed C-terminal deletion mutation after progression disease. Radiation before rituximab administration may also be related to mutagenesis of CD20 gene. Because one patient showed C-terminal deletion mutation before immunotherapy, we also need to consider clonal selection of CD20 after R-CHOP therapy. Moreover, microsatellite instability is known to be one of the mechanisms of gene mutation (28). Although microsatellite instability was examined as the cause of CD20 mutation in four patients with the C-terminal deletion mutation, it was not observed in their lymphoma cells (data not shown). Because two of these patients had received radiotherapy before rituximab therapy, radiation may have caused the CD20 mutation before treatment. However, some researchers have found that rituximab-resistant cells with low CD20 levels of rituximab have the same CD20 gene sequence as that of sensitive cells (29, 30), suggesting that various or other mechanisms may contribute to CD20 down-regulation.

Although we found the C-terminal deletion mutation clones more often in patients with disease progression than at initial diagnosis, C-terminal deletion mutation was also strongly related to a shortening of the drug-free duration. Clinical prognostic factors for B-cell malignant lymphoma are well described and include age, Ann Arbor clinical stage, hemoglobin level, number of affected lymph nodes, and lactate dehydrogenase level (31, 32). Moreover, DNA microarray analysis implicates expression of several genes, including BCL2, BCL6, and ZAP70, as denoting poor prognosis in B-cell malignant lymphoma (33–36). However, there has been no report about gene mutations within molecular markers of lymphoma, such as the CD20 gene. Here, we have presented the first data showing that a CD20 gene mutation is related to a decline in CD20 expression and poor patient outcome. Because the mutation was detected in patients with disease progression, a more sensitive assay should be developed to detect CD20 mutations at initial diagnosis.

In conclusion, we found that C-terminal deletion mutations of CD20 were related to relapse/resistance after rituximab therapy, and screening for these mutations should be done in patients with disease progression after partial remission.

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Yasuhito Terui, Yuji Mishima, Natsuhiko Sugimura, et al.


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