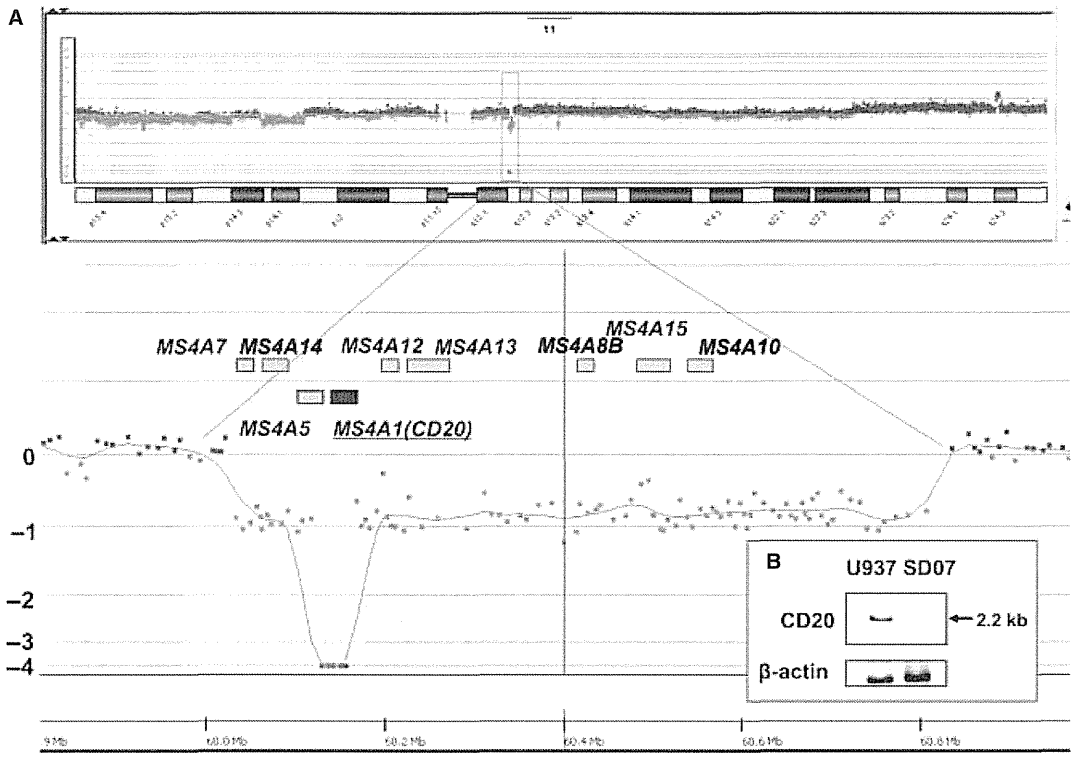

Figure 3 Genomic alteration of the *CD20* gene in SD07. (A) Genomic DNA was isolated from SD07 cells using a Quick Gene-800 (Fujifilm, Tokyo, Japan). The Human Genome CGH Microarray 244K (Agilent Technologies, Palo Alto, CA, USA) was used for copy number measurement. Bone marrow DNA of this patient without infiltration of lymphoma cells was used as a reference. (B) Southern hybridization of the *CD20* gene of SD07 using CD20 Exon 1 as a probe. Twenty micrograms of genomic DNA was digested with EcoRI. A monocytic leukemia cell line, U937, was used as a positive control.

We could not analyze precisely the status of the *CD20* gene in lymphoma cells at the time this patient was diagnosed because the tissue that remained from the needle biopsy was not sufficient for DNA analysis. Therefore, it is still not known whether the B-cell lymphoma cell clones that proliferated with CD20 deletion at relapse appeared *de novo* after immune-chemotherapy with rituximab or whether the B-cell lymphoma cell clones with pre-existing (minor) *CD20* gene deletion were selected and then proliferated as a result of the immune-chemotherapy. In this case, an immunohistochemical analysis with the CD20 antibody at diagnosis was not helpful in determining the origin of the *CD20* gene-deleted lymphoma cell at relapse, because more than 80% of the lymphoma cells were positive for CD20 in the initial diagnostic biopsy.

The copy number loss of 11q12 of DLBCL is uncommon as analyzed by CGH array, suggesting that the reduced expression of CD20 observed in the case of DLBCL at diagnosis is not likely to be the result of deletion of the *CD20* gene (7). Loss of 11q12 also was not reported in the relapse in DLBCL, although this particular CGH analysis was performed before the introduction of rituximab in B-cell lymphoma chemotherapy (8).

The alteration of the *CD20* gene is reported to be infrequent at CD20-negative relapse in B-cell lymphoma (3, 5). However, the previous observation, obtained primarily through PCR analysis, possibly missed the deletion-related mutation of the *CD20* gene in lymphoma tissue at relapse. This study suggests the alteration of the *CD20* gene needs to be studied carefully in diffuse large B-cell lymphoma cases involving the loss of expression of CD20.

Loss of CD20 expression is important not only because of the potential loss of a therapeutic target at relapse/disease progression in the case of DLBCL, but because it is often associated with a poor prognosis of the patient at relapse. In newly diagnosed cases of DLBCL, reduced expression of CD20 at diagnosis is associated with a low chance of survival (9). Studies showed reduced expression of CD20 in DLBCL is associated with chemorefractory phenotypes, such as those that are positive for CD5 and bcl-2(9). Down-regulation of CD20 was observed in double-hit lymphoma cases involving *c-myc* and *BCL2* or *BCL6*, which are also known as chemorefractoy phenotypes (10). In SD07, array CGH showed several genomic regions with copy number loss that is possibly involved in a refractory phenotype of SD07. These include 10q23 (phosphatase and tensin homolog, *PTEN*) (Figure S5). The deregulated PTEN/Akt/PI3K pathway is important in B-cell lymphomagenesis (11). Because PTEN is involved in the anti-proliferative effect mediated by

the anti-CD20 antibody (12), deletion of both CD20 and PTEN might contribute to disease progression in this case. In SD07, we found copy number loss in the 11q12 region. The region contains the membrane-spanning 4A gene family (MS4A1) cluster, which includes *MS4A8B*, *MS4A13*, *MS4A12*, *MS4A5*, *MS4A14*, *MS4A10*, *MS4A7*, and *MS4A15* in addition to *MS4A1* (*CD20*). Of these, MS4A7 and MS4A8B are expressed in B-cell lymphoma cell lines (13). Members of the MS4A gene family, as well as CD20, have recently been reported to be involved in the cell cycle progression of lymphocytes and signal transduction of the immune response (14, 15). The loss of function(s) of the MS4A family gene(s), in addition to CD20, may be involved in tumorigenicity in SD07.

Molecular pathways that regulate expression of CD20 and how CD20 affects the phenotype in B-cell lymphoma remains to be studied, although CD20 physiologically has a function in B-cell activation, regulation of B-cell growth, and transmembrane calcium flux. We are now studying the functional role(s) of expression of CD20 in lymphomagenesis in the case of DLBCL using SD07 as a model.

In summary, we have established a DLBCL cell line with loss of CD20 expression because of the homozygous deletion of the CD20 gene. This represents refractory B-cell lymphoma cells that appeared at CD20-negative relapse after rituximab-containing chemotherapy. Deletion of the *CD20* gene is a molecular mechanism of CD20-negative relapse in a subset of DLBCL. Because SD07 represents chemo-refractory DLBCL with the loss of CD20 expression, an analysis of SD07 is expected to provide a therapeutic rationale to overcome DLBCL relapse following immuno-chemotherapy treatment with rituximab.

Acknowledgements

The authors would like to thank Mrs. Atsuko Nagasawa and Mrs. Mutsumi Oouchi for their technical assistance in this study. This work was supported in part by the High-Technology Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology.

References

1. Hiraga J, Tomita A, Sugimoto T, Shimada K, Ito M, Nakamura S, Kiyoi H, Kinoshita T, Naoe T. Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance. *Blood* 2009;**113**:4885–93.
2. Maeshima AM, Taniguchi H, Nomoto J, Maruyama D, Kim SW, Watanabe T, Kobayashi Y, Tobinai K, Matsuno Y. Histological and immunophenotypic changes in 59 cases of B-cell non-Hodgkin's lymphoma after rituximab therapy. *Cancer Sci* 2009;**100**:54–61.
3. Johnson NA, Leach S, Woolcock B, deLeeuw RJ, Bashashati A, Sehn LH, Connors JM, Chhanabhai M, Brooks-Wilson A, Gascoyne RD. CD20 mutations involving the rituximab epitope are rare in diffuse large B-cell lymphomas and are not a significant cause of R-CHOP failure. *Haematologica* 2009;**94**:423–7.
4. Sugimoto T, Tomita A, Hiraga J, Shimada K, Kiyoi H, Kinoshita T, Naoe T. Escape mechanisms from antibody therapy to lymphoma cells: downregulation of CD20 mRNA by recruitment of the HDAC complex and not by DNA methylation. *Biochem Biophys Res Commun* 2009;**390**:48–53.
5. Sar A, Perizzolo M, Stewart D, Mansoor A, Difrancesco LM, Demetrick DJ. Mutation or polymorphism of the CD20 gene is not associated with the response to R-CHOP in diffuse large B cell lymphoma patients. *Leuk Res* 2009;**33**:792–7.
6. Terui Y, Mishima Y, Sugimura N, *et al.* Identification of CD20 C-terminal deletion mutations associated with loss of CD20 expression in non-Hodgkin's lymphoma. *Clin Cancer Res* 2009;**15**:
2523–30.
7. Bea S, Zettl A, Wright G, *et al.* Diffuse large B-cell lymphoma subgroups have distinct genetic profiles that influence tumor biology and improve gene-expression-based survival prediction. *Blood* 2005;**106**:3183–90.
8. Berglund M, Enblad G, Flordal E, *et al.* Chromosomal imbalances in diffuse large B-cell lymphoma detected by comparative genomic hybridization. *Mod Pathol* 2002;**15**:807–16.
9. Johnson NA, Boyle M, Bashashati A, *et al.* Diffuse large B-cell lymphoma: reduced CD20 expression is associated with an inferior survival. *Blood* 2009;**113**:3773–80.
10. Wu D, Wood BL, Dorer R, Fromm JR. "Double-Hit" mature B-cell lymphomas show a common immunophenotype by flow cytometry that includes decreased CD20 expression. *Am J Clin Pathol* 2010;**134**:258–65.
11. Miletic AV, Anzelon-Mills AN, Mills DM, Omori SA, Pedersen IM, Shin DM, Ravetch JV, Bolland S, Morse HC 3rd, Rickert RC. Coordinate suppression of B cell lymphoma by PTEN and SHIP phosphatases. *J Exp Med* 2010;**207**:2407–20.
12. Gupta P, Goldenberg DM, Rossi EA, Chang CH. Multiple signaling pathways induced by hexavalent, monospecific, anti-CD20 and hexavalent, bispecific, anti-CD20/CD22 humanized antibodies correlate with enhanced toxicity to B-cell lymphomas and leukemias. *Blood* 2010;**116**:3258–67.
13. Liang Y, Tedder TF. Identification of a CD20-, FcepsilonRIb-eta-, and HTm4-related gene family: sixteen new MS4A family members expressed in human and mouse. *Genomics* 2001;**72**:119–27.
14. Donato JL, Ko J, Kutok JL, Cheng T, Shirakawa T, Mao XQ, Beach D, Scadden DT, Sayegh MH, Adra CN. Human HTm4 is a hematopoietic cell cycle regulator.
J Clin Invest 2002;**109**:51–8.
15. Xu H, Yan Y, Williams MS, Carey GB, Yang J, Li H, Zhang GX, Rostami A. MS4a4B, a CD20 homologue in T cells, inhibits T cell propagation by modulation of cell cycle. *PLoS ONE* 2010;**5**:e13780.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PCR assay for immunoglobulin (Ig) kappa (κ) chain gene rearrangement.

Figure S2. The karyotype of the CD20-negative DLBCL cell line and SD07.

Figure S3. The expression of CD20 mRNA and protein in SD07.

Figure S4. Effects of rituximab on the cell proliferation of B-cell lymphoma cell lines Daudi, N8, TK, and SD07.

Figure S5. Genomic alteration of the *PTEN* gene in SD07.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.