Discussion

In clinical practice, CD20 expression abnormalities have been reported. Johnson et al. [18] reported that 43 out of 272 (16%) patients with diffuse large B-cell lymphoma (DLBCL) showed reduced CD20 expression using FCM analysis at the time of initial diagnosis, and that the survival rate of this phenotype was significantly lower than that of patients with CD20-positive phenotype. Furthermore, we previously reported that a CD20-negative phenotypic change after using rituximab resulted in resistance to salvage chemotherapies with or without rituximab [6,7]. We observed that all of these patients died of disease progression within 1 year after the diagnosis of CD20-negative transformation, suggesting that the CD20-negative phenotype may be related to the poor prognosis. From these findings, we realized the importance of investigating the mechanisms of downmodulation of CD20 expression to explore overcoming strategies including salvage combination chemotherapies with anti-CD20 antibodies.

In this study, we firstly investigated the effect of 5-Aza-dC on RRBL1 cells. DNMT1 protein reduction was observed 1 day after adding 5-Aza-dC, followed by temporal upregulation of CD20 protein expression (Fig. 1B). This phenomenon suggested that CpG demethylation of the *MS4A1* promoter region was a result of DNMT1 depletion. But interestingly, significant CpG islands were not located at the promoter, suggesting that *MS4A1* activation by 5-Aza-dC was not regulated directly by *MS4A1* promoter methylation.

The next hypothesis we investigated was that expression of transcription factors, which is critical for *MS4A1* expression, was regulated by the methylation status of the promoter DNA. We analyzed the protein expression level of IRF4/Pu.1, and only a modest upregulation was observed. Furthermore, the ChIP assay showed that IRF4/Pu.1 recruitment to the *MS4A1* promoter was fairly stable in the presence or absence of 5-Aza-dC and TSA (Fig. 3B). On the other hand, Sin3A–HDAC1 recruitment and histone deacetylation was observed in the absence of epigenetic drugs. Because previous reports have indicated that HDACs form large protein complexes, such as Sin3 [26], NuRD/Mi-2 [27], and N-CoR/SMRT co-repressor complexes [26,28], and are recruited to the specific promoter by transcription factors, we analyzed whether the Sin3A–HDAC1 complex interacts with IRF4 in RRBL1 cells. Using an IP assay, we observed that HDAC1 interacts with Sin3A but not with IRF4 (Fig. 4B). We also analyzed the recruitment of the proteins N-CoR, HDAC3, and TBLR1 (transducin β-like protein 1 relating protein), which are all expressed in the same co-repressor complex *in vivo* [21–23,26,28], to the promoter region using the ChIP assay. Significant recruitment of these proteins was not seen in this assay (data not shown).

Thus, these findings suggest that, (1) *MS4A1* repression is not directly regulated by methylation of its promoter and (2) transcription factors other than IRF4 recruit the Sin3A–HDAC1 co-repressor complex to the *MS4A1* promoter to repress transcription through histone deacetylation. Our previous report [6] showed that treatment with TSA without 5-Aza-dC upregulates CD20 expression in RRBL1 cells within 1 day, suggesting that the activity of HDAC may be more critical for *MS4A1* expression than the activity of DNMTs. One explanation for why 5-Aza-dC can stimulate *MS4A1* expression is that the expression of some transcription factors, whose expression is critical for CD20 expression, may be regulated by CpG methylation of the gene promoters. The maximal effect of 5-Aza-dC on CD20 protein expression was seen at 3 days after treatment with 5-Aza-dC, which is consistent with this hypothesis. The knockdown of endogenous DNMT1 using the siRNA technique may help explain the importance of DNMT1 for *MS4A1* repression. On the other hand, the possibility that CpG islands in *MS4A1* that affect its expression are in a location that is relatively remote (~5 kb) from the transcription start site cannot be excluded. Further investigation is needed.

In our study, the efficiency of stimulating CD20 protein expression in CD20-negative transformed cells using epigenetic drugs is not complete (Fig. 2B). As we showed previously [7], this efficiency may not be sufficient to overcome resistance to rituximab. Using the newer generation humanized-anti-CD20 monoclonal antibodies, such as ofatumumab [29], GA-101 [30], and others, which have higher antibody binding capacity with CD20 and/or a higher CDC/ADCC activity, may help overcome the resistance. We also anticipate the use of those therapies in combination with epigenetic drugs such as HDAC and/or DNMT inhibitors. Further investigation is still needed.

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Presentation and management of intravascular large B-cell lymphoma

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Intravascular large B-cell lymphoma (IVLBCL) is a rare disease entity of non-Hodgkin lymphoma according to the current WHO classification. This rare form of B-cell lymphoma is characterised by selective growth of tumour cells in the lumina of small vessels of various organs. Strange characteristics of IVLBCL, including the absence of marked lymphoadenopathy and the usually aggressive clinical behaviour, result in the delay of timely and accurate diagnosis and fatal complications. Thus, the prognosis of IVLBCL is extremely poor. The success achieved with the anti-CD20 chimeric monoclonal antibody, rituximab, represents an important milestone in the clinical practice of B-cell lymphoma. An advantage of adding rituximab to conventional chemotherapies has been shown, in the process of increasing our understanding of the clinical and pathological manifestations for IVLBCL. This Review describes the cutting edge of research on IVLBCL, and discusses the unsolved issues from biological and clinical perspectives to provide a better understanding of this rare lymphoma.

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Introduction

Intravascular large B-cell lymphoma (IVLBCL) is a rare disease entity of malignant lymphoma, characterised by the selective growth of lymphoma cells within the lumina of vessels. This type of lymphoma was first reported in 1959 by Pfleger and Tappeiner[1] as "angioendotheliomatosis proliferans systemisata" and was considered to be endothelial in origin.[2] In 1982, Ansell and colleagues[3] suggested a lymphoid origin by showing surface immunoglobulin on neoplastic cells. Leucocyte common antigen on neoplastic cells was subsequently reported in 1985,[4,5] and the lymphoid nature of this entity was confirmed in 1986 by Wick and co-workers.[6] Since the first description, other historical names for IVLBCL have included angioendotheliomatosis proliferans systemica, malignant angioendotheliomatosis, neoplastic angioendotheliosis, intravascular lymphomatosis, angioendotheliotropic (intravascular) lymphoma (Kiel classification), angiotropic large-cell lymphoma (Luke-Collins classification), and diffuse large B-cell lymphoma (Revised European American Lymphoma classification).[7] According to the last WHO classification,[8] IVLBCL was defined as a rare subtype of extranodal diffuse large B-cell lymphoma, and has been classified as an independent disease entity in the recent revision of classifications.[9]

Definition of IVLBCL

According to the current WHO classification, IVLBCL is defined as an extranodal B-cell lymphoma characterised by tumour involvement in the lumina of vessels, especially capillaries, with the exception of larger arteries and veins (figure 1).[9] Lymphoadenopathy is usually absent in IVLBCL. Peculiar characteristics of this lymphoma result in tumour cells involving all types of organs, such as bone marrow, the CNS, skin, lung, adrenal gland, liver, kidney, spleen, thyroid, pituitary gland, and gastrointestinal tract, among others, with insult to organs resulting from tumour infiltration.[10–18] The cells of origin for IVLBCL are not completely understood, but might be postgerminal-centre cells, on the basis of the presence of somatic mutations in immunoglobulin heavy chain variable region (VH) gene analyses.[19]

Morphology and immunophenotype

Neoplastic lymphoid cells mainly exist in the lumina of small or intermediate-sized vessels in various organs. Tumour cells are large with prominent nucleoli and frequent mitotic figures. According to the recent consensus meeting of expert haemopathologists and clinicians for IVLBCL, rare cases of this disease show cells with anaplastic features or of smaller size, and minimum extravascular location of neoplastic cells can sometimes be seen.[20] Sinusoidal involvement occurs in the liver, spleen, and bone marrow (figure 2).[21–23] Malignant cells are occasionally detected in peripheral blood.[24] Most IVLBCL tumour cells immunophenotypically express B-cell-associated antigens.[9] Several case

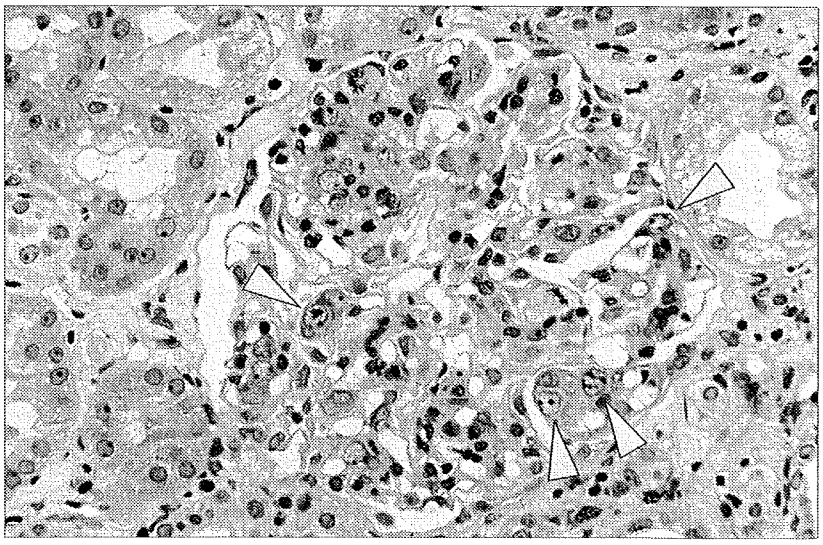

Figure 1: **Intravascular large B-cell lymphoma (IVLBCL)**
Arrows indicate IVLBCL tumour cells. Tumour cells with large nucleoli and mitosis involve small vessels in glomerular capillaries (haematoxylin and eosin stain, original magnification ×200).

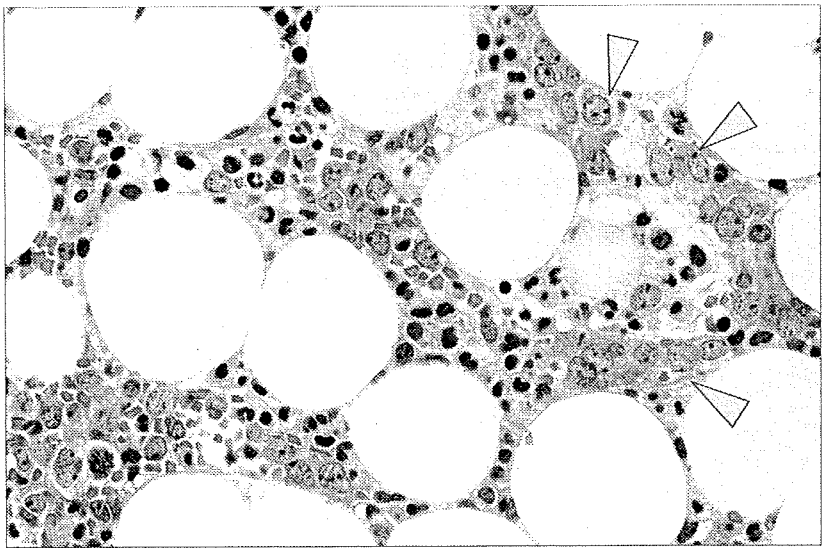

Figure 2: **Bone marrow specimen from a patient diagnosed with intravascular large B-cell lymphoma**
Arrows show tumour cells. Tumour cells show intrasinusoidal patterns (haematoxylin and eosin stain, original magnification ×200). Reproduced with permission from reference 20.

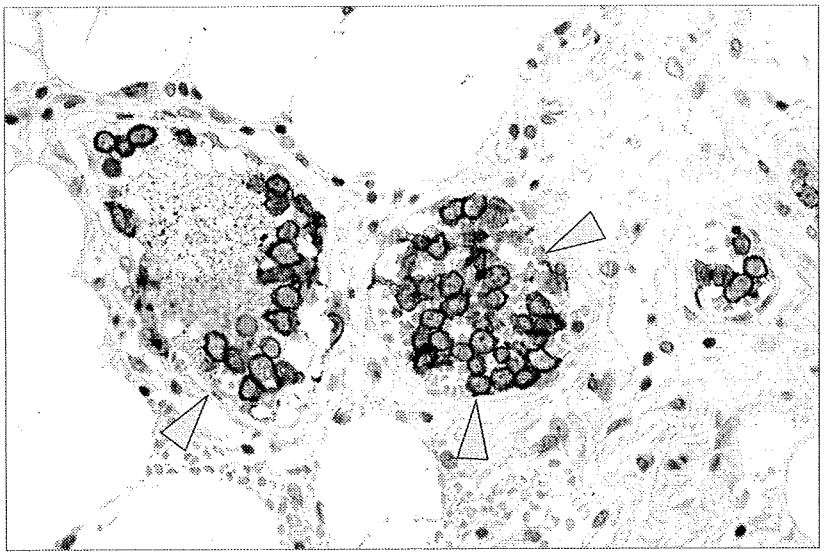

Figure 3: **Immunohistochemical staining for intravascular large B-cell lymphoma**
Arrows show CD20+ lymphoma cells. CD20+ lymphoma cells are increasing in lumina of small vessels in splenic hilar veins (CD20 immunostaining, original magnification ×200).

reports have shown intravascular T-cell or natural-killer-cell lymphoma confirmed by immunophenotypical analysis,[25–27] and some of these cases have been associated with Epstein–Barr virus infection.[28–30] Cases possessing T-cell or natural-killer-cell antigens are thought to represent different disease entities from IVLBCL. Almost all patients with IVLBCL express CD20 (figure 3), and CD5 expression has been shown to be positive in 36 of 96 patients (38%) and CD10 in 12 of 96 patients (13%) with IVLBCL.[17] CD5-positive cases of IVLBCL are accompanied by expression of MUM-1, and categorised as a non-germinal-centre type.[17,31] In a recent analysis of cases in Asian countries, all cases of CD10-negative IVLBCL were categorised as non-germinal-centre types.[17] By comparison, 20% of cases of IVLBCL were classified as germinal-centre B-type in an immunophenotypical analysis of cases in European countries.[21] These findings suggest that almost all cases of IVLBCL are classified as non-germinal-centre types. This finding is shared with other extranodal lymphomas, such as diffuse large B-cell lymphoma of the CNS,[32] but whether this is related to the strange biological behaviour of extranodal distributions remains to be clarified. This characteristic of IVLBCL leads us to postulate that the pathogenetic mechanism in IVLBCL is associated with adhesion molecules or homing receptors necessary for migration into extravascular regions. A small number of reports regarding adhesion molecules have been published. Ponzoni and colleagues[33] suggested that tumour cells of IVLBCL do not express CD29 (beta-1 integrin) or CD54 (intercellular adhesion molecule [ICAM]-1) on the basis of an immunohistochemical analysis of a small number of cases. Other groups have reported that CD11a was related to the presence of tumour cells in vessel lumina.[34,35] However, biological evidence for tumour cells lodging in the lumina of vessels remains insufficient. The difficulties of obtaining sufficient patient samples of IVLBCL and immunohistochemical staining for adhesion molecules have contributed to this paucity of information.

Cytogenetic and genetic features

Characteristics of the cytogenetic and genetic features of IVLBCL remain incompletely understood. Previous reports have suggested that immunoglobulin genes are clonally rearranged.[19,34] In a small case series, structural aberrations in chromosomes 1, 6, and 18, especially 1p (in four of six patients) and trisomy 18 (in four of six patients), were shown.[36] Another study showed a patient with chromosomal abnormalities including t(11;14)(q13;q32), indicating an abnormality of cyclin D1, which was confirmed by karyotype analysis of tumour cells and interphase fluorescence in-situ hybridisation (FISH).[37] In our recent cohort analysis, 48 of 84 (57%) available patients showed cytogenetic abnormalities.[24] Pathognomonic cytogenetic abnormalities have not been reported in IVLBCL until recently.

Clinical features

IVLBCL typically occurs in elderly patients. In our recent cohort, with clinical outcomes updated in December, 2007, the median age of the 106 patients was 67 years (range 34–84), and 76 of 106 patients (72%) were older than 60 years.[24] IVLBCL is equally common in men and women, with a male:female ratio of 1·3:1 (56% *vs* 44%). Tumour cells can involve any systemic organ, with various systemic symptoms, such as fever of unknown origin, general fatigue, marked deterioration in performance status, and neurological alteration. Identifying this disease in patients with such heterogeneous and

non-specific symptoms can be difficult for many clinicians. However, as a result of the recent developments in diagnostic procedures and the spread of knowledge about IVLBCL, the frequency of patients diagnosed with this disease antemortem is believed to be increasing.

Presenting symptoms at initial diagnosis in our cohort compared with those in a European series of patients with IVLBCL are summarised in table 1.[24,38] The clinical manifestations differ between these two distinct geographical areas. In Asian countries, IVLBCL predominantly accompanies a haemophagocytic syndrome known as Asian-variant IVLBCL.[24] In European countries, IVLBCL predominantly involves skin and the CNS, especially as a "cutaneous variant" limited to the skin.[38] In both cohorts, the most common symptom was fever (78 of 106 [74%] and 17 of 38 [45%], respectively), with general fatigue also noted in many patients.[24,38] Furthermore, loss of appetite was the most common symptom in patients with gastrointestinal symptoms in our cohort.[24] The fact that fever, general fatigue, and loss of appetite without lymphadenopathy, all of which are extremely common symptoms, are the most prevalent symptoms of IVLBCL directly contributes to the difficulty of accurate and timely diagnosis.

Neurological symptoms at initial diagnosis were noted in 25% of patients in our cohort.[24] Although this proportion is smaller than that noted in the European series,[12,38,39] neurological symptoms are important symptoms leading to accurate diagnosis. Neurological symptoms noted in patients with IVLBCL are heterogeneous, and alteration of consciousness, motor and sensory deficits, seizure, paresis, dementia, intentional tremor, disorientation, and gating disturbance were recorded in our cohort.[24] Patients with IVLBCL often present with more than one neurological symptom. Various causes of these neurological symptoms can be considered. Invasion of tumour cells into the CNS or peripheral nerves, which leads to impaired microcirculation of these nerves, might represent a major cause of these symptoms. High fever and metabolic aberration with disease progression might be considered as important causes of alterations of consciousness, especially in elderly patients with IVLBCL. However, the specificity of neurological testing is low.[40] In terms of the neuroimaging features of IVLBCL, radiological findings are non-specific, and features of IVLBCL and CNS vasculitis can be identical. Computed tomography (CT) is generally considered non-diagnostic and magnetic resonance imaging (MRI) can show non-specific hyperintensity of white-matter lesions suggestive of small-vessel ischaemic disease or demyelination.[41] However, imaging modalities detect CNS involvement in only half of patients with IVLBCL with neurological symptoms.[41] Therefore, suspecting the existence of IVLBCL on the basis of these neurological symptoms is difficult. Nevertheless, clinicians should consider IVLBCL as one of the differential diagnoses in patients with neurological symptoms and fever, and MRI should be done in patients with suspected IVLBCL.

	Asian cohort[24] (n=106), n (%)	European cohort[38] (n=38), n (%)
Fever	78 (74)	17 (45)
General fatigue	28 (26)	6 (16)
Gastrointestinal symptoms	21 (20)	2 (5)
Neurological symptoms	26 (25)	13 (34)
Dyspnoea	21 (20)	1 (3)
Oedema	11 (10)	2 (5)
Urinary tract symptoms	1 (1)	3 (8)
Skin eruptions	6 (6)	15 (39)

Table 1: **Presenting symptoms at initial diagnosis of intravascular large B-cell lymphoma**

Skin lesions differ substantially between the Asian and European series.[24,38] In European countries, 10 of 38 patients (26%) with IVLBCL were diagnosed with the cutaneous variant of the disease.[38] Skin lesions showed heterogeneous morphology and distribution, including painful indurate erythematous eruption, violaceous plaques, cellulitis, solitary plaques, and ulcerated nodules.[38] A few studies have reported generalised telangiectasia as a clinical manifestation of IVLBCL.[42,43] In our recent cohort, two patients showed skin eruption at initial onset of disease and an additional four patients developed skin eruptions before diagnosis.[24] Furthermore, 13 patients without skin eruptions were diagnosed with skin involvement of tumour cells on the basis of random skin biopsies. To accurately assess the incidence of skin involvement in Asian countries, further studies are needed in which random skin biopsies are used. Such a wide difference between Asian and European countries, in terms of neurological symptoms and skin eruptions, is deemed to reflect differences in clinical manifestations between the two groups.

As a characteristic of Asian-variant IVLBCL, haemophagocytic syndrome represents the most relevant clinical manifestation.[44,45] Haemophagocytic syndrome was noted in 63 of 106 (59%) of patients in our recent cohort.[24] However, haemophagocytosis was absent in patients with IVLBCL in a European series.[12] To our knowledge, only small numbers of patients with IVLBCL and haemophagocytic syndrome have been reported in USA and France.[22,46] This difference between distinct geographical areas is intriguing. Although the reason underlying the prevalence of haemophagocytosis in Asian series is unknown, this difference might exist as a result of ethnic differences associated with the production of inflammatory cytokines, including interferon-γ, tumour necrosis factor-α, and interleukin-1β, and soluble interleukin-2 receptor (sIL2R), leading to systemic inflammatory response.[47,48] In fact, sIL2R levels were significantly higher in patients with Asian-variant IVLBCL than in patients with non-Asian-variant IVLBCL in our series.[24]

	Number of patients, n (%)
Anaemia (Hb <110 g/L or RBC <350×10⁹/L)	72 (68)
Thrombocytopenia (platelet count <100×10⁹/L)	62 (58)
Leucocytopenia (WBC <4·0×10⁹/L)	29 (27)
Increased lactate dehydrogenase (>ULN)	104 (98)
Hypoalbuminaemia (<30 g/L)	62 of 102 (61)
Bilirubin (>15 mg/L)	18 of 102 (20)
Creatinine (>15 mg/L)	11 of 102 (13)
C-reactive protein (>50 mg/L)	61 of 104 (59)
Soluble interleukin-2 receptor (>5000 U/mL)	63 of 96 (66)

Hb=haemoglobin. RBC=red-blood-cell count. WBC=white-blood-cell count. ULN=upper limit of normal.

Table 2: **Abnormalities on blood examination in the Asian cohort (n=106)[24]**

Other organ involvement including liver, lung, and kidney should be mentioned. These organs might be potential targets for diagnostic biopsies. In our cohort, liver dysfunction (bilirubin >25·7 μmol/L) was noted in 18 of 102 patients (18%) and renal dysfunction (serum creatinine concentration >132·6 μmol/L) was noted in 11 of 102 patients (13%).[24] In fact, 14 of 81 patients (17%) were diagnosed with IVLBCL by liver biopsy.[17] Respiratory symptoms and lesions, including dyspnoea, hypoxia, and pulmonary involvement of tumour cells were noted in 35 of 106 patients (33%).[24] In a study from a single institute, hypoxia diagnosed by blood-gas analyses was noted in 11 of 12 patients, irrespective of the existence of clinical symptoms.[49] This finding suggests that potential pulmonary involvement of IVLBCL is more common than generally recognised. Furthermore, a few studies have reported endocrine dysfunction with IVLBCL.[50] A case of hypopituitarism with IVLBCL and gradual reversal of pituitary dysfunction after immunochemotherapy has been reported.[10]

Staging

Ferreri and colleagues[12] reported stage IV disease accounting for 76% of cases of prevailing cutaneous variant of IVLBCL in the European series.[12] In another report, 12 of 30 patients (40%) with in-vivo diagnosis of IVLBCL showed stage IE disease, according to the Ann Arbor staging system.[38] In our cohort, all patients with IVLBCL were classified with stage IV disease.[24] In view of the fact that autopsy cases showed disseminated disease and random skin biopsies showed tumour-cell involvement, despite an absence of apparent skin eruptions, IVLBCL can spread widely in the absence of apparent signs of involvement of various organs.

Abnormalities on blood examination

In our cohort, all patients with IVLBCL showed various abnormalities on blood examination (table 2).[24] The most common abnormality was increased concentration of serum lactate dehydrogenase, which was noted in 104 of 106 patients (98%) in our cohort and in 33 of 39 (69%) in the European series.[12,24] Haematological abnormalities are common; in our cohort, 72 of 106 patients (68%) had anaemia (haemoglobin concentration <110 g/L or red-blood-cell count <350×10⁹ per L), 62 of 106 (58%) had thrombocytopenia (platelet count <100×10⁹ per L), and 29 of 106 (27%) had leucocytopenia (white-blood-cell count <4·0×10⁹ per L).[24] In the European series, anaemia (haemoglobin concentration <120 g/L) and leucocytopenia (white-blood-cell count <4·0×10⁹ per L) have been noted to the same extent, but thrombocytopenia (platelet count <150×10⁹ per L) was less frequent, reflecting the existence of haemophagocytosis in the Asian cohort.[38] SIL2R level was substantially high (>5000 U/mL) in 63 of 96 patients (66%) in our series.[24] Hypoalbuminaemia (<30 g/L) is common in Asian series (62 of 102 patients [61%]), but not in the European series (six of 33 [18%]).[12,24] Substantially increased aminotransferase concentrations are also uncommon, with only 20% of patients showing increased aminotransferase concentrations above the upper limit of normal in our cohort.[24]

Diagnosis of IVLBCL

Organ biopsies are mandatory for the accurate diagnosis of IVLBCL. Timely and accurate diagnosis is extremely important for patients with this disease, because appropriate treatment can improve clinical outcomes.[24] However, no standard procedure for accurate diagnosis of IVLBCL has been established. In view of the fact that tumour involvement can occur in any organ and identification of tumour cells is relatively easy from pathological specimens, the organs selected by the physician for biopsy are key to accurate diagnosis, when physicians suspect the existence of this type of disease from symptoms such as fever or increased serum lactate dehydrogenase concentration. In Asian cohorts, the most relevant diagnostic site seems to be the bone marrow.[17] Tumour-cell involvement with intrasinusoidal patterns in bone-marrow biopsy specimens is common (figure 2). In fact, a previous report showed a role for repeated bone-marrow biopsies in accurate diagnosis.[13] Furthermore, reports on random skin biopsies are promising for prompt diagnosis.[42,51–53] The fact that tumour cells are identified from random skin biopsies, not only in European series in which the cutaneous variant is prevalent, but also in Asian series in which the cutaneous variant is rare, is remarkable. In our recent cohort, the increasing number of random skin biopsies has led to an increase in the number of patients who can be diagnosed with IVLBCL by this diagnostic procedure.[24] However, some patients present with negative findings from random skin biopsies. Further large-scale studies are needed to clarify the significance of random skin biopsies, because the number of studies on this new diagnostic procedure is currently too small to confirm its usefulness. Renal biopsies under CT guidance have also been reported.[54] However, the severe complication of intraperitoneal

haemorrhage on renal biopsy has occurred in patients with IVLBCL (Shimada K, unpublished data). In our cohort, disseminated intravascular coagulation had developed by the time of diagnosis in 18 of 72 patients (25%).[24] Physicians should therefore pay close attention to laboratory findings when organ biopsies are done. Regarding pulmonary investigations, 11 patients in our cohort received lung biopsies and were diagnosed with pulmonary involvement of tumour cells.[24] Transbronchial lung biopsies might be useful for diagnosis, especially in patients with respiratory symptoms.

In the assessment of patients with non-Hodgkin lymphoma, 18[F]-fluorodeoxyglucose (FDG)-PET has emerged as a powerful functional imaging tool.[55] Use of FDG-PET has been recommended for initial staging and post-treatment assessment in diffuse large B-cell lymphoma.[56] However, the role of FDG-PET in IVLBCL remains controversial. In view of the aggressive and progressive clinical behaviour with increased lactate dehydrogenase concentrations in almost all cases, rapid sugar consumption due to high cell turnover in IVLBCL would be expected.[57,58] FDG-PET in this disease is therefore expected to be useful, as it is in diffuse large B-cell lymphoma. In fact, a small number of case reports have shown the usefulness of FDG-PET for diagnosis of IVLBCL, when the disease was clinically suspected, especially due to fever of unknown origin.[59–61] However, we assessed the accuracy of FDG-PET in detecting disease involvement of IVLBCL.[57] In our study, FDG-PET was able to detect only two of seven pathologically confirmed lesions as positive FDG-PET findings and the number of tumour cells in pathological specimens tended to be high when FDG-PET and biopsy findings matched.[57] This finding suggests that the number of tumour cells per volume might be lower for IVLBCL, with selective growth of tumour cells in the lumina of small vessels, than for nodal lymphoma. Although diagnostic accuracy of FDG-PET in IVLBCL might be lower compared with nodal diffuse large B-cell lymphoma, FDG-PET could detect useful information leading to accurate diagnosis and prediction of severe complications, especially pulmonary complications as described in the toxicity section, which could not be obtained using conventional diagnostic methods.[62,63] Further studies are needed to establish the role of FDG-PET in this disease.

Treatment of IVLBCL

Clinical outcomes of IVLBCL were extremely dismal before the rituximab era. One of the important reasons for such dismal prognosis was the difficulty of timely and accurate diagnosis. In previous reports, about half of patients were diagnosed post mortem and the effect of steroid therapies was tentative.[64] In fact, we still occasionally see and hear of patients diagnosed with IVLBCL post mortem. In 1994, DiGiuseppe and colleagues[65] reported clinical outcomes for ten patients at Johns Hopkins Hospital (MD, USA). In their report, four

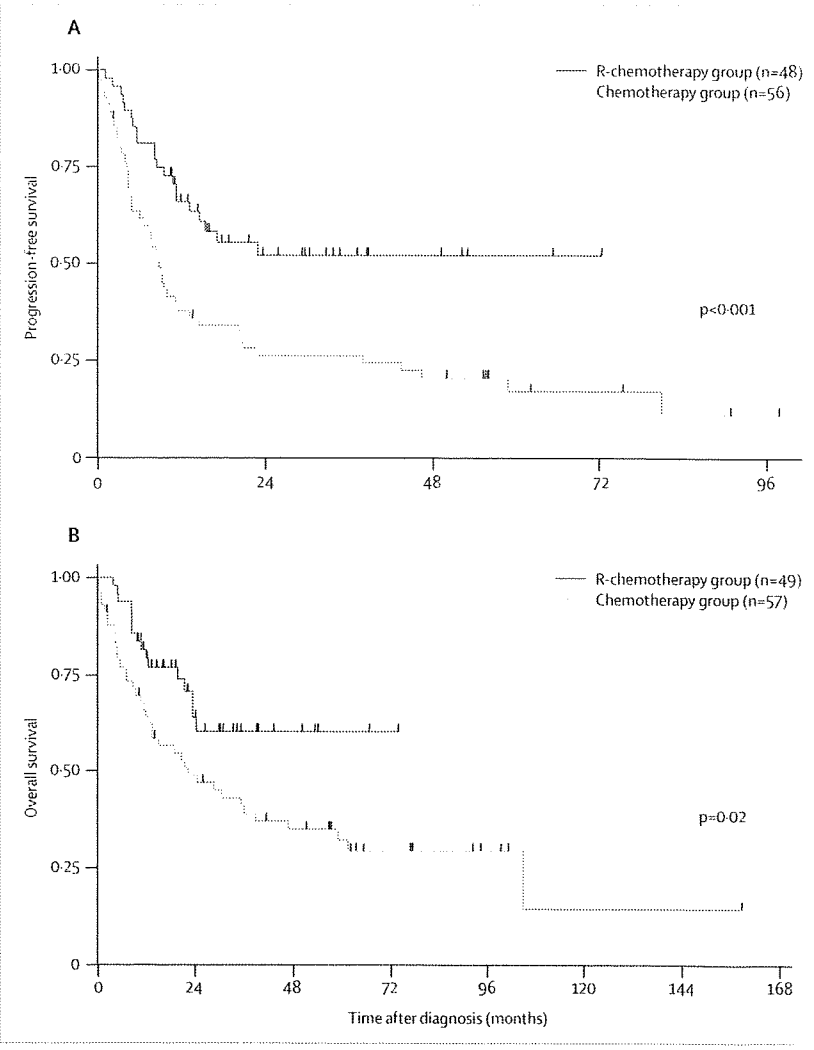

Figure 4: **Progression-free survival (A) and overall survival (B) for patients who received chemotherapy with rituximab (R-chemotherapy) or without rituximab (chemotherapy) in our recent cohort**

of the ten patients received chemotherapy for IVLBCL, and two of the four patients receiving chemotherapy survived for around 4 years after diagnosis. The median survival of the remaining six patients without chemotherapy was 3 months (range 1–19). Ferreri and co-workers[66] reported clinical outcomes for 22 patients who received chemotherapy in 2004. 3-year overall survival was 33%. In 2008, we reported a retrospective analysis of clinical outcomes for 106 patients receiving chemotherapy with or without rituximab.[24] We assessed the clinical outcomes of 57 patients receiving chemotherapy without rituximab and 49 patients receiving chemotherapy with rituximab. With a median follow-up of 18 months (range 1–95), progression-free survival (PFS) and overall survival at 2 years in patients receiving chemotherapy with rituximab were 56% and 66%, respectively.[24] In patients who received chemotherapy without rituximab, PFS and overall survival at 2 years were 27% and 46%, respectively.[24]

Search strategy and selection criteria

Data for this Review were identified by searches of PubMed using the following search terms: "lymphoma", "intravascular and large-cell lymphoma", "angiotrophic lymphoma", "angiotrophic lymphomatosis", "hemophagocytic syndrome", and "FDG-PET". Reports from correspondence in the *Journal of Clinical Oncology* were included only when related directly to previously published work. The definition of IVLBCL was referred to by "WHO Classification Tumours of Haematopoietic and Lymphoid Tissues". Except for papers regarding the history of IVLBCL, only reports published between January, 1984, and May, 2009, were included. Only articles published in English were used.

In the follow-up data,[24,67] with a median follow-up of 26 months (range 10–74) in patients receiving chemotherapies with rituximab, PFS and overall survival at 3 years were 53% and 60%, respectively (Kazuyuki Shimada [unpublished data]; figure 4). This finding suggests that clinical outcomes for rituximab-containing chemotherapy remain constant for more than 2 years of follow-up. Ferreri and colleagues[68,69] also showed improved clinical outcomes for patients with IVLBCL who received immunochemotherapies in European countries.[68,69] In their study, overall survival at 3 years was 81% in 33 patients who received chemoimmunotherapies.[68] Both of the retrospective studies from Asian and European countries suggested improvement of clinical outcomes of IVLBCL in the rituximab era.

Several reports have shown efficacy for high-dose therapy with autologous stem-cell support (ASCT) in patients with IVLBCL.[70–74] In our study, ten of 14 patients who received ASCT did so in the first remission.[24] Seven patients survived without relapse after ASCT until the end of the study.[24] In view of the fact that about 80% of patients with IVLBCL were classified into the high-risk group according to international prognostic index (IPI), ASCT in first remission might represent a useful treatment option.[73] In our cohort, median age at diagnosis in patients with IVLBCL was 67 years (range 34–84), and 55% of patients were more than 65 years of age.[24] This meant that most patients with IVLBCL were ineligible for ASCT.

Toxic effects of immunochemotherapy

Toxic effects associated with administration of rituximab should be mentioned. The characteristic of tumour-cell growth in the lumina of small vessels is deemed to have major ramifications for severe immunoreactions with rituximab infusion. In fact, severe pulmonary complications related to rituximab infusion as an initial treatment have been reported.[63] In our study, 14 (29%) of 49 patients developed adverse events related to rituximab infusion (fever [n=12]; hypotension [n=9]; hypoxia [n=5]; and chills [n=3]). Grade 3 hypoxia was noted in one patient (2%) who received rituximab infusion on the first day of treatment.[24] Mild adverse effects, including chills, fever, and hypotension were also noted in three of 34 patients (12%), and severe adverse events, including pulmonary failure and coma after the first course of treatment, were noted in two of 34 patients (6%) in a recent European cohort.[68] Therefore, delaying rituximab administration by several days in the first course of treatment might be a useful option to avoid severe infusion reactions to rituximab, especially in patients with severe organ damage, including hypoxia related to pulmonary involvement.

Prognostic factors

Ferreri and colleagues[66] reported favourable prognostic factors, including performance status 1, disease limited to the skin, stage I disease, and use of chemotherapy in European countries. Murase and colleagues[17] also reported use of chemotherapy as a favourable prognostic factor in an Asian series. Both cohorts included a small number of patients using immunochemotherapies, so these findings represent prognostic factors in the pre-rituximab era. In our study, use of rituximab was identified as a favourable prognostic factor for IVLBCL.[24] Because almost all patients with IVLBCL were categorised in the high-risk group according to IPI in our cohort,[24] the established prognostic index might not be useful. Up to now, predictive factors that are useful for risk-stratification of patients have not been established.

Conclusion and future perspective

With recent developments in the understanding of disease manifestation, the accuracy of diagnosis for IVLBCL seems to be increasing. Recent retrospective analyses of Asian and European cohorts suggest that clinical outcomes of IVLBCL have improved in the immunochemotherapy era.[24,67,68,69] Although the difficulty of obtaining tumour cells from patients is related to research difficulties, in terms of biological aspects, we expect further improvements in the understanding of this rare type of lymphoma. In terms of treatment strategy, whether the rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) regimen is a sufficient treatment strategy should be investigated. In view of the high incidence of CNS relapse and involvement at initial diagnosis, CNS-oriented therapy, including high-dose methotrexate, might be warranted.[68] Further investigations are mandatory to achieve better outcomes for patients with IVLBCL.

Contributors

KS contributed to the idea for the paper, literature search, and writing. TK helped with the idea, writing, financial support, and critical review. TN helped with writing, financial support, and critical review, and SN contributed to the writing and critical review.

Conflicts of interest

KS, TK, and SN have received honoraria from Chugai Pharmaceutical Ltd (Tokyo, Japan). TK has received a grant for research from Chugai Pharmaceutical Ltd and Zenyaku Kogyo (Tokyo, Japan). TN declared no conflicts of interest.

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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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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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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 rebiopsy was performed in 19 patients. Of those 19, 5 (26.3%; diffuse large B-cell lymphoma [DLBCL], 3 cases; DLBCL transformed from follicular lymphoma, 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 reverse-transcription–polymerase chain reaction (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. (Blood. 2009;113: 4885-4893)

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.[1,2] Many favorable results using combination chemotherapy with rituximab for both CD20-positive de novo and relapsed low-grade and aggressive B-cell non-Hodgkin lymphoma have been reported in recent years.[3-7] In Japan, rituximab has also been used since September 2001 for patients with follicular lymphoma (FL), indolent lymphoma, and mantle cell lymphoma (MCL). In addition, since September 2003 in Japan, indications for using rituximab were expanded to include diffuse large B-cell lymphoma (DLBCL), further demonstrating the significant effectiveness of rituximab for B-cell lymphoma compared with conventional chemotherapies without rituximab.[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 (reviewed by Smith et al[9]),[10-19] 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,[16,20-31] indicating that this phenomenon after the use of rituximab may not be rare. Although these reports contain important information from clinical experiences, the frequency of occurrence and detailed molecular biologic 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,[32] and the mechanisms of the CD20-negative change were analyzed in these cells. 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), immunohistochemistry (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 biologic 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 (29.0%) of whom showed relapse/progression. Among these 36 patients, 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, as well

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Table 1. CD20-positive B-cell malignancies treated with rituximab in Nagoya University Hospital

	No. of patients	Disease status at rituximab therapy		Response, RD/PD	Resampling of tumor tissue	CD20 expression, +/±/−
		1st	RD/PD			
DLBCL	51	45	6	13	6	3/1/2
FL	43	26	17	13	7	5/0/2
Nodal marginal zone BCL or MALT	8	6	2	2	2	2/0/0
Burkitt or Burkitt-like	5	5	0	4	2	2/0/0
Mediastinal large B-cell	4	2	2	1	0	0
Intravascular large B-cell	4	4	0	2	1	1/0/0
Mantle cell	4	3	1	1	1	1/0/0
Lymphoplasmacytic	3	3	0	0	0	0
CLL/SLL	2	1	1	0	0	0
Total cases	124	96	28	36	19	14/1/4
Probability (%)				36/124 (29.0)	19/36 (52.5)	5/19 (26.3)

DLBCL indicates 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; and +/±/−; positive/decreased/negative.

as 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, 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.[33] 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 years) 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, computed tomography (CT), magnetic resonance imaging (MRI), and ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET). Resampling of tumors at the time of relapse/progression and pathologic analysis of 19 patients was performed. The patients' responses to chemotherapies were evaluated using the International Working Group criteria.[34]

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.[32,35] Briefly, we used mouse anti-CD20 (L26; Dako, Carpinteria, CA), anti-CD10 (Novocastra Laboratories, Newcastle-upon-Tyne, United Kingdom), and anti-CD79a monoclonal antibodies (Dako) for IHC, and mouse anti-CD20 (B2E9; Beckman Coulter, Fullerton, CA) 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 percentages of negative and positive cells from FCM were 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) and used for further polymerase chain reactions (PCRs). 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). Genomic DNA PCR was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA) 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-upper (U), 5′-GCT CTT CCT AAA CAA CCC CT-3′; exon 3-lower (L), 5′-CAT GGG ATG GAA GGC AAC TGA C-3′; exon 4-U, 5′-TGC TGC CTC TGT TCT CTC CC-3′; exon 4-L, 5′-CTG CAC CAT TTC CCA AAT GGC T-3′; exon 5-U, 5′-CTC CAT 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 GAT TAT G-3′; exon 6-L, 5′-CCT GGA GAG AAA TCC AAT CTC A-3′; exon 7-U, 5′-GTC TCC TGT ACT AGC AGT TC-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) and were sequenced using a DNA auto sequencer (ABI PRISM 310; Applied Biosystems). 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-transcription–polymerase chain reaction

A blood RNA extraction kit (QIAGEN) was used to isolate total RNA from tumor cells. cDNA was prepared as reported previously.[36] For reverse-transcription–polymerase chain reaction (RT-PCR) analyses of CD10, CD19, CD20, and β-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 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 ATG-3′; β-actin-U, 5′-TCA CTC AAG ATC CTC A-3′; and β-actin-L, 5′-TTC GTG GAT GCC ACA GGA C-3′. Semiquantitative RT-PCR with AmpliTaq Gold was performed as described previously.[32] Quantitative RT-PCR was carried out using TaqMan PCR (ABI PRISM 7000; Applied Biosystems) as previously described.[32,36]

Immunoblot analysis

Cells (~5 × 10^5) were lysed in 100 μL lysis buffer (50 mM tris(hydroxymethyl)aminomethane [Tris]-HCl [pH 8.0], 1.5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ester)-*N,N,N′,N′*-tetraacetic acid [EGTA], 5 mM KCl, 10% glycerol, 0.5% Nonidet P-40 [NP-40], 300 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol [DTT], and a Complete Mini protease inhibitor tablet [Roche Applied Science, Indianapolis, IN]). After centrifugation at 10 000*g* for 10 minutes, the supernatants were placed in new tubes and 100 μL of 2× sodium dodecyl sulfate (SDS) sample buffer was added. After boiling for 5 minutes, samples were

Table 2. CD20-negative RD/PD after treatment with rituximab-containing combination chemotherapy

UPN	Age/ sex	Diagnosis on admission	Status	Chemo Regimen	Total ritux	Duration until CD20−	Diagnosis (RD/PD)	Patho source	CD20 expression				Survival after CD20−
									FCM	IHC	RT	CDS mutation	
1	65/M	DLBCL	2 rel	R-salvage	8	2M	DLBCL	BM	−	−	N.A.	S97F [2/16]	6M†
2	37/ F	DLBCL	1 diag	R-CHOP	8	9M	DLBCL	BM	±*	±†	N.A.	V247I [2/10]	4M†
3	75/M	DLBCL	1 diag	R-CHOP	7	10M	DLBCL	BM, CF	−	−	↓	WT	11M†
4	42/M	FL G1	2 rel	R-CHOP	4	23M	DLBCL	BM	−	−	↓	WT	11M†
5	52/M	FL G2	3 rel	R-cMOPP	14	81M	DLBCL	BM, LN‡	−	−	↓	WT	8M†

Status indicates disease status of those patients at the first treatment with rituximab; rel, relapse; duration until CD20−, duration (in 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 pathologic analysis; BM, bone marrow; CF, cerebral fluid; LN, lymph node; RT, RT-PCR; N.A., not available; ↓, down-regulated; CDS, coding sequences; and survival after CD20−, duration from CD20-negative change until death.

*19% of tumor cells were $CD20^+$.

†30% of tumor cells showed $CD20^+$.

‡Lymph node sample obtained at autopsy.

separated by SDS–polyacrylamide gel electrophoresis (PAGE). Immunoblotting was carried out as described previously[32,37] using goat polyclonal anti-CD20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) 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[32] and primary lymphoma cells (5×10^5) were cultured in 6-well dishes in RPMI 1640 medium containing 10% fetal bovine serum (FBS) for 24 hours with or without 5-aza-2′-deoxycytidine (5-Aza; Sigma-Aldrich, St Louis, MO) at a final concentration of 100 mM. The cells were then washed twice with RPMI 1640 medium containing 10% FBS, and incubated for more than 2 days in the same medium without 5-Aza. Cells were harvested and used for total RNA and protein extraction.

Antibody-dependent cell-mediated cytotoxicity assay in vitro

Antibody-dependent cell-mediated cytotoxicity (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% to 20% FBS. Each cell line (2.0×10^5 cells) was labeled with 100 μCI (3.7 MBq) of $Na_2{}^{51}CrO_4$ (PerkinElmer 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. ^{51}Cr-labeled target cells were divided into aliquots in 96-well plates (10^4 cells/well). Effector PBMC cells (5×10^5 cells/well) were then added to each well in the presence or absence of rituximab (0 to 31.25 μg/mL) and incubated for 4 hours at 37°C. Supernatants were obtained after a brief centrifugation and measured on a γ-ray counter (PerkinElmer). ^{51}Cr-labeled target cells without antibodies were lysed completely by NP-40 (2% final concentration) and used 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))] × 100%, where cpm indicates counts per minute.

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; relapse/progression of disease [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 resampled 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 resampled 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/m^2) 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. Histologic 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, and other factors. More patients must be studied to further analyze this apparent trend.

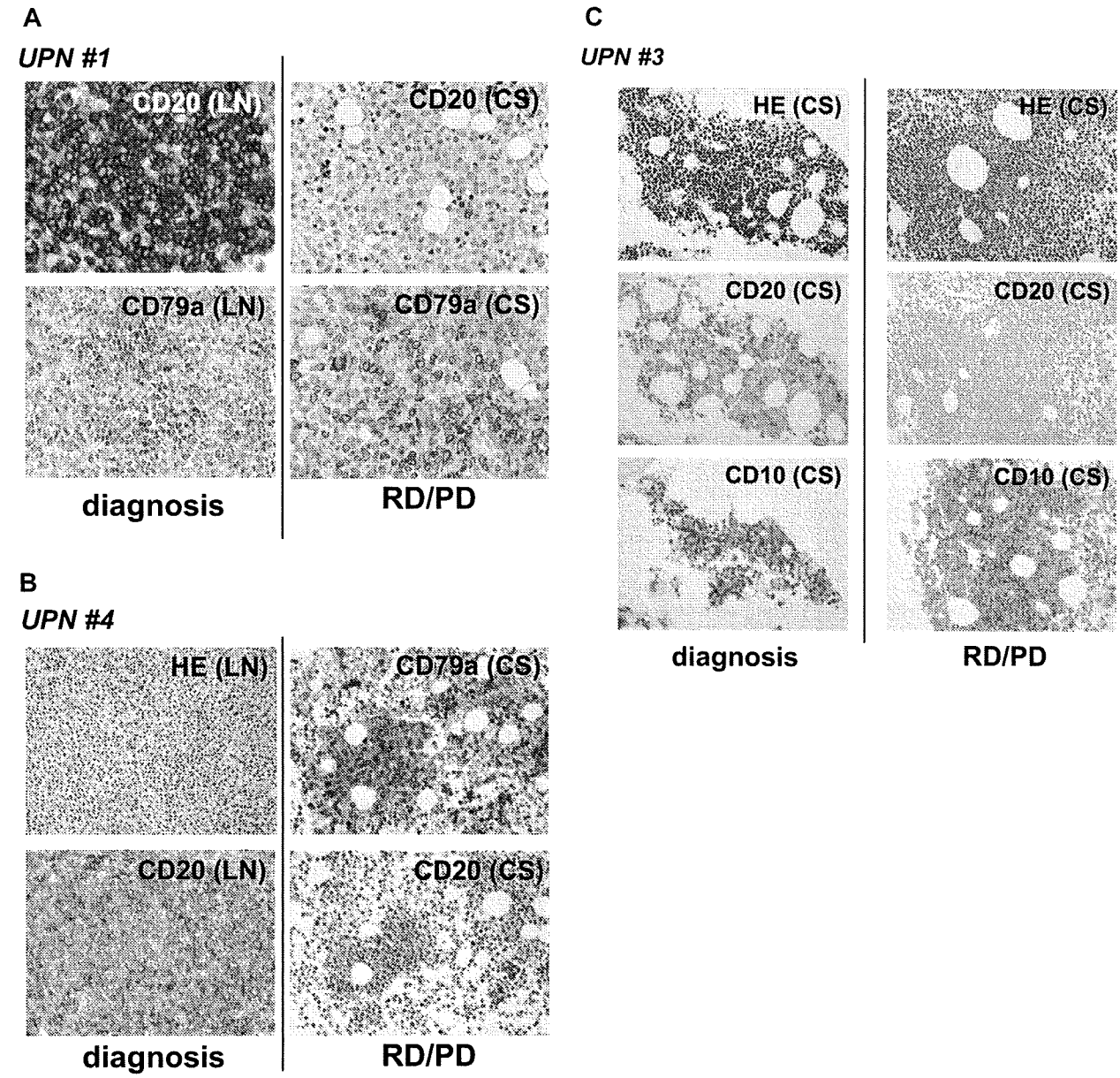

Figure 1. CD20 protein–negative phenotypic changes in CD20-positive B-cell lymphoma patients after treatment with rituximab-containing chemotherapy. Tissue samples (LN, lymph nodes; CS, bone marrow clot section) obtained from UPNs 1 (A), 4 (B), and 3 (C) in Table 2 were analyzed by IHC using anti-CD20, anti-CD79a, and anti-CD10 antibodies. Anti-CD79a antibody was used 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 magnifications, ×400 (A) and ×200 (B,C) (Olympus BX51TF microscope, Olympus, Tokyo, Japan, and Nikon DS-Fi1 camera, Nikon, Tokyo, Japan). HE indicates hematoxylin and eosin staining; and RD/PD, relapse/progression of disease.

Table 3. Response against salvage chemotherapies for the 36 patients who showed relapse or PD after rituximab-containing chemotherapies.

	CD20 expression		
Response	–	+	N.D.
CR	0	5	3
PR	0	1	3
SD	0	1	1
RD/PD	0	2	2
Death	5	5	8
Total cases	5	14	17

These outcomes were evaluated in July, 2007.

CR indicates complete remisson; PR, partial response; SD, stable disease; RD/PD, relapse/progression; and N.D., Not determined.

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 unique patient number (UPN) 1 and valine 247 to isoleucine (V247I; GTT → ATT) in UPN 2 were confirmed in 2 clones each 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

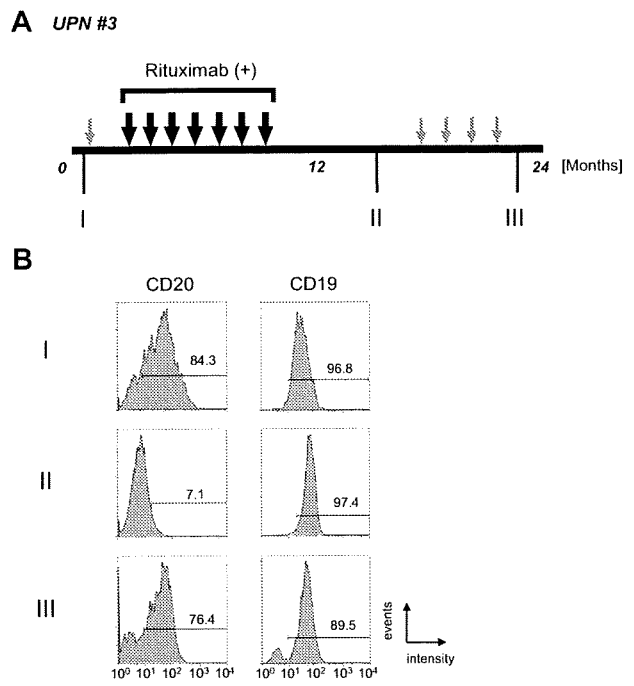

Figure 2. Alteration of CD20 protein expression on B-cell lymphoma cells during disease progression. (A) The clinical course of UPN 3 is depicted briefly. Large black arrows and smaller gray arrows indicate one course of combination chemotherapy with or without rituximab, respectively. Rituximab (375 mg/m^2 each) was administered 7 times. During the patient's 24-month clinical course, tumor cells were harvested at stages I, II, and III from lymph nodes, bone marrow, peripheral blood, and/or cerebral fluid. (B) FCM analysis using anti-CD20 and anti-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.

(CD20-negative; Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). 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 stage II, but was reversed at stage III. At stage 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). Semiquantitative 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,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[38,39] may be able to stimulate the *CD20* transcription. First, we examined *CD20* transcription after treatment with 5-Aza using primary tumor cells derived from cerebral fluid of UPN 3 at stage II in Figure 2A, which

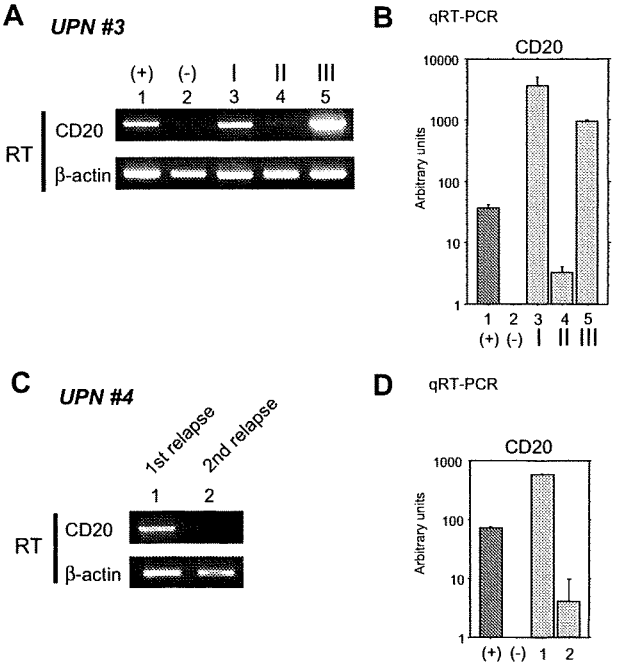

Figure 3. Alteration of CD20 mRNA expression in B-cell lymphoma cells during the clinical course. (A) RT-PCR (RT) was performed using total RNA from the same tumor cells as in Figure 2B (UPN 3 in Table 2). As positive and negative controls, total RNA from Raji and 293T cells was used (lanes 1 and 2), respectively. I, II, and III (lanes 3-5) correspond to the clinical stages depicted in Figure 2A. (B) Quantitative RT-PCR was performed using the same RNA as in panel 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 by + and −, respectively.

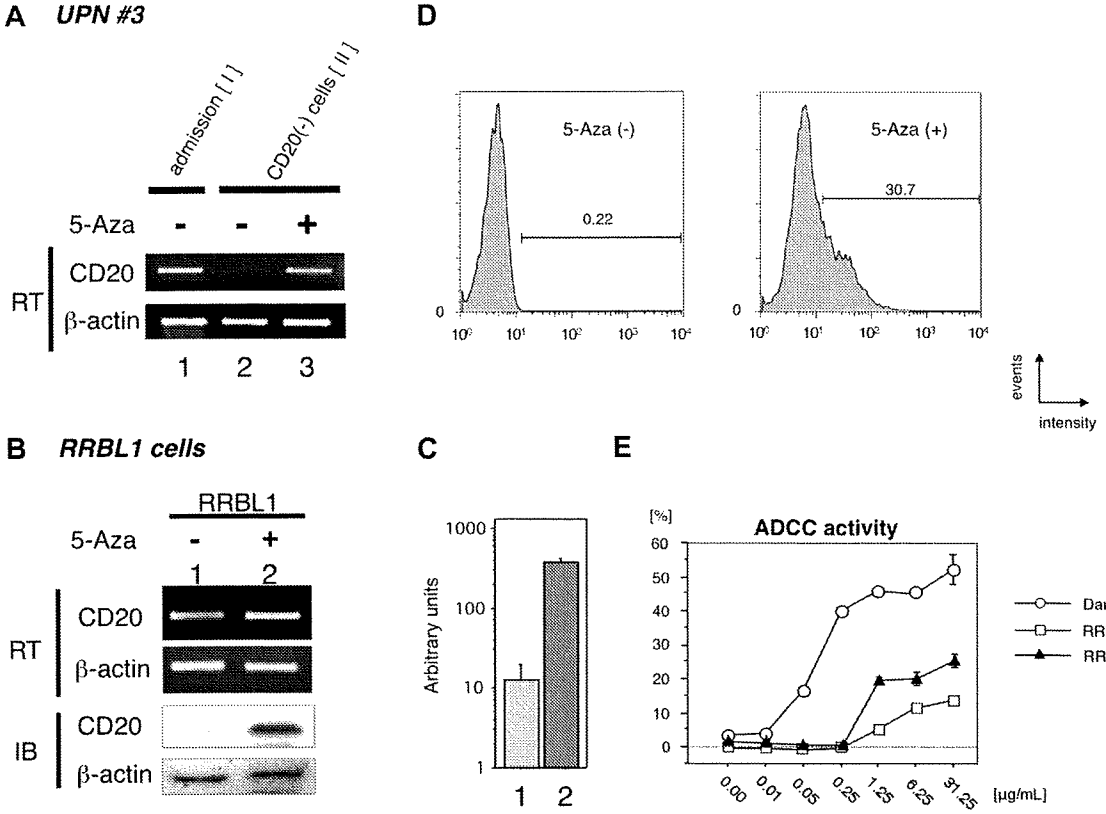

Figure 4. Restoration of CD20 mRNA and protein expression by treatment with the DNA methyltransferase inhibitor 5-Aza. (A) Primary B-cell lymphoma cells, which showed a CD20 protein-negative phenotype from UPN 3, were incubated with or without 5-Aza. Total RNA was prepared, and semiquantitative RT-PCR 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 used in lane 1. (B) The CD20 protein-negative B-cell lymphoma cell line RRBL1,[32] 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, semiquantitative 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 ^{51}Cr-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 (○), but not in RRBL1 cells (□), 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 (▲). Error bars indicate plus or minus 1 standard deviation.

showed a CD20-negative phenotype. As a CD20-positive control, lymphoma cells at the initial diagnosis from the same patient were used (Figure 4A lane 1). After treatment with 5-Aza in vitro, significant stimulation of CD20 expression was observed (Figure 4 lane 3).

Previously, we established the CD20-negative B-cell lymphoma cell line RRBL1,[32] which was derived from CD20-negative tumor cells in peripheral blood from a 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,C). CD20 protein expression induction was also confirmed by immunoblotting (Figures 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-Aza in vitro

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 ^{51}Cr-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 with 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 antitumor 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. Importantly, not all patients who show RD/PD demonstrate resistance to rituximab. In fact, some patients were sensitive to retreatment with rituximab-containing salvage chemotherapies (data not shown). We may need to define "rituximab resistance" more carefully by monitoring each patient's clinical course.

A CD20-negative phenotypic change was observed in 26.3% of patients for whom tumor resampling (rebiopsy) was carried out (Table 1). We generally perform rebiopsies when tumor progression becomes very aggressive or when the manner of tumor expansion significantly changes during clinical observation. If we had carried out resampling 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 5 patients died from their disease progression within 1 year after showing a CD20-negative transformation (Tables 2,3). This observation may indicate that a loss of CD20 expression is partly related to poor prognosis. In our study, however, patients' backgrounds varied (eg, age, sex, pathologic findings, chemotherapy 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) may instead 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 expression,[41] may contribute to the aberrant *CD20* transcriptional regulation. We analyzed the methylation status of cytosine guanine dinucleotides (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 suggested the possibility that both *CD20* gene expression (at the pre- and posttranscriptional level) and protein down-regulation are related to the loss of CD20 protein expression after treatment with rituximab in vitro, resulting in rituximab resistance.[10] In addition, down-regulation of CD20 protein surface expression by internalization into the cytoplasm was also observed in some specific cases.[16,42] 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 of the patients showed a CD20-negative phenotypic change were 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 2 cases, as shown in Table 2. These mutations led to amino acid alterations, including S97F and V247I, which are located at the second transmembrane domain and the C-terminal intracellular domain, respectively. A recent report suggested that neither site is recognized directly by rituximab.[43] Although it is possible that these alterations led 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 complement-dependent cytotoxicity (CDC) 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 posttranslational modification of the CD20 protein, abnormalities in the CD20 signal transduction pathway, antiapoptotic mechanisms of tumor cells, or aberrant metabolism of rituximab.[9,44,45] The detailed mechanisms of these and other possibilities are still unclear.

Finally, in the specific cases reported herein, 5-Aza can stimulate CD20 mRNA and protein expression, resulting in the restoration of rituximab sensitivity in vitro. The DNA methyltransferase inhibitors 5-azacytidine and 5-Aza have been used in patients suffering from hematologic malignancies such as myelodysplastic syndrome.[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 patients. Further analysis of patients' primary cells and in vivo analysis using mouse xenograft lymphoma models are required.

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Authorship

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 pathologic analyses; H.K. and T.K. analyzed data, designed experiments, and interpreted data; and T.N. supervised experiments and wrote the paper.

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