resulted in a continued increase in the risk of allergic reactions. Therefore, to lessen the risk of allergic reactions to oxaliplatin, it seems prudent to limit the treatment period to 4–4.5 months (8 or 9 cycles of FOLFOX4; total dose, 680–765 mg/m²). This recommendation allows patients enough time to achieve the maximum tumor response, as it helps them to avoid severe neurotoxicity[26] and diminishes the risk of allergic reactions.

In previous studies, the median time to tumor response was 9 weeks in patients treated with FOLFOX4[2]; patients receiving oxaliplatin experienced severe neurotoxicity after a cumulative dose of 780–850 mg/m². In our study, nine patients developed grade 2 neurotoxicity after a median of 4 months (8 cycles of FOLFOX4) and a median total dose of 676 mg/m² (range, 340–1,105 mg/m²) without experiencing any severe allergic reactions. We still are investigating predictors of allergic reactions to oxaliplatin that may enable us to avoid severe reactions.

In conclusion, severe reactions to oxaliplatin generally are manageable if appropriate treatment is provided immediately. Oxaliplatin-based chemotherapy will continue to be first-line treatment for various cancers. Therefore, an appropriate strategy to prevent such allergic adverse reactions should be devised and assessed in a larger trial.

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Soluble interleukin-2 receptor retains prognostic value in patients with diffuse large B-cell lymphoma receiving rituximab plus CHOP (RCHOP) therapy

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Background: Soluble interleukin-2 receptor (SIL-2R) is known to be a prognostic parameter in patients with diffuse large B-cell lymphoma (DLBCL) receiving cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) therapy. However, its prognostic value has not been well known since the introduction of rituximab.

Patients and methods: We retrospectively evaluated the prognostic impact of SIL-2R in 228 DLBCL patients, comparing 141 rituximab-combined CHOP (RCHOP)-treated patients with 87 CHOP-treated patients as a historical control.

Results: Patients with high serum SIL-2R showed significantly poorer event-free survival (EFS) and overall survival (OS) than patients with low SIL-2R in both the RCHOP group (2-year EFS, 66% versus 92%, $P < 0.001$; OS, 82% versus 95%, $P = 0.005$) and the CHOP group (2-year EFS, 40% versus 82%; OS, 61% versus 90%, both $P < 0.001$). Multivariate analysis including the five parameters of International Prognostic Index (IPI) and two-categorized IPI revealed that SIL-2R was an independent prognostic factor for EFS and OS in the RCHOP group as well as in the CHOP group.

Conclusions: Our results demonstrate that SIL-2R retains its prognostic value in the rituximab era. The prognostic value of SIL-2R in DLBCL patients receiving rituximab-combined chemotherapy should be reassessed on a larger scale and by long-term follow-up.

Key words: diffuse large B-cell lymphoma, rituximab, soluble interleukin-2 receptor

introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma [1]. It takes an aggressive clinical course and comprises a heterogeneous group of lymphomas in terms of morphology, phenotype, molecular biology and clinical behavior. Up to now, the International Prognostic Index (IPI) has been the most widely used predictive model for patients with DLBCL treated with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) [2]. On the other hand, soluble interleukin-2 receptor (SIL-2R) has also been investigated as a prognostic factor, and several studies have demonstrated that a high level of SIL-2R before treatment is associated with both a low remission rate and poor prognosis [3–8].

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SIL-2R is the soluble form of interleukin-2 receptor (IL-2R). IL-2R is expressed on the cell membrane of lymphocytes and plays important roles in their activation and proliferation [9]. It is composed of at least three glycoprotein chains: α (55 kDa), β (75 kDa) and γ (64 kDa). Each subunit is able to bind to the ligand independently with either low (IL-2Rα) or intermediate (IL-2Rβ and γ) affinity. It is now possible to examine the expression of the soluble-type α subunit [10]. The soluble IL-2Rα chain is induced and expressed only after mononuclear cell (T cell, B cell, monocyte, and natural killer cell) activation [11, 12]. Therefore, activated T and B cells have elevated levels of SIL-2R.

Although the CHOP regimen has been the mainstay of treatment for aggressive lymphomas for several decades [13], treatment outcome has significantly improved with the introduction of rituximab (an anti-CD20 chimeric antibody) in both young and elderly patients [14–17]. Since the introduction of rituximab, several prognostic factors have been reevaluated. Sehn et al. [18] recently reevaluated five prognostic

factors and demonstrated that the IPI remained predictive; they proposed a revised IPI in which DLBCL patients are classified into very good (no IPI risk factors), good (one to two risk factors) and poor (three to five risk factors) categories. In contrast, BCL2, BCL6 and immunohistochemically defined germinal center (GC) phenotype have been reported to have no prognostic value when rituximab is added to chemotherapy [19–24]. Other clinical factors or biomarkers identified in patients receiving CHOP therefore need to be reassessed in patients treated with CHOP combined with rituximab.

Up to now, the prognostic value of SIL-2R in RCHOP has not been investigated. The aim of the present study was to retrospectively reassess the prognostic value of SIL-2R in DLBCL patients receiving RCHOP as compared with CHOP alone and to investigate whether or not this factor still influences the outcome of DLBCL.

patients and methods

patient characteristics

In the present study, we reviewed the medical records of patients with CD20-positive DLBCL who received CHOP with or without rituximab as a first-line therapy at the Cancer Institute Hospital from January 2000 to December 2006 and were followed until January 2008. The study protocol and sampling were approved by the Institutional Review Board of the Cancer Institute Hospital. Informed consent for retrospective analysis and additional immunophenotypic analysis and gene rearrangement studies was obtained.

Patients were analyzed if they were older than 18 years and had a performance status (PS) of zero to three according to the criteria of the European Cooperative Oncology Group. Patients were excluded if they had clinically relevant cardiac diseases or positivity for antibodies against HIV-1 or 2. Patients with primary mediastinal large B-cell lymphoma, primary CNS lymphoma and primary testicular lymphoma were also not included in this study.

The disease stage was evaluated according to the Ann Arbor staging system. All patients had undergone staging investigations, including physical examinations, blood and serum analysis, bone marrow aspiration and biopsy and computed tomography of the neck, chest, abdomen and pelvis. Magnetic resonance imaging was used for evaluation of involved organs in the head and neck. The following clinical and laboratory data were available at the time of diagnosis: age, sex, serum lactate dehydrogenase level, PS, presence of B symptoms, clinical stage and number of extranodal sites. This allowed the IPI scores to be determined in the studied patients. Patients were categorized into either a low-risk group (IPI score, 0–2) or a high-risk group (IPI score, 3–5). Response to initial therapy was evaluated according to the Cheson criteria [25].

treatment

In both the CHOP and RCHOP groups, CHOP chemotherapy was given triweekly at a standard dose. Patients with stages IB–IV received six cycles, and patients with stage IA three cycles, of CHOP chemotherapy followed by radiotherapy for the involved field. After incorporation of rituximab into the CHOP regimen in February 2004, patients were treated with RCHOP regimen, in which rituximab was administered at a standard dose of 375 mg/m^2 once weekly for 8 weeks concurrently with triweekly CHOP, as described previously [26].

chemical studies

The serum SIL-2R levels were determined using a sandwich enzyme-linked immunosorbent assay kit (Cell-free Interleukin-2 Receptor Test Kit, T Cell Science, Cambridge, MA) using two mAbs against distinct two different epitopes of the p55 alpha-chain of the IL-2R complex. Serum SIL-2R was considered 'high' when higher than the median and 'low' when lower than the median.

pathological studies

Biopsy samples collected before treatment were fixed in formalin, embedded in paraffin, sliced and stained with hematoxylin and eosin for morphological analysis. For diagnosis of DLBCL, immunohistochemical analysis was carried out using the dextran-polymer method (EnVision+; Dako, Glostrup, Denmark) with mAbs against CD5, CD10, CD20, Ki67, BCL2, BCL6 and MUM1 in most cases and with CyclinD1 to exclude the possibility of a pleomorphic variant of mantle cell lymphoma when the lymphoma was CD5 positive. Patients with a small-cell component implying transformation from low-grade/indolent B-cell lymphoma were excluded. All the samples were reviewed by an expert hematopathologist (KT).

statistical analysis

Basic characteristics of the CHOP group and RCHOP group were compared by Fisher's exact test. Event-free survival (EFS) was calculated from the date of diagnosis to the date of documented disease progression, relapse or death from any cause or to the stopping date. Overall survival (OS) was calculated from the date of diagnosis until death from any cause or the last follow-up. If the stopping date was not reached, the data were censored at the date of the last follow-up evaluation. Survival curves were estimated by the Kaplan–Meier method, and overall differences were compared by the log-rank test. Log-rank test was carried out according to SIL-2R, two-categorized IPI for the two treatment groups. To estimate the unbiased prognostic impacts of SIL-2R on EFS and OS, Cox proportional hazards analysis was applied. First, we conducted univariate Cox analysis for SIL-2L, all IPI factors and dichotomized IPI and then we carried out multivariate Cox analysis adjusted for SIL-2R and each of the IPI risk factors, with final adjustment for SIL-2R and dichotomized IPI. Only factors that were associated with at least a trend toward significance in the univariate analysis (unadjusted P value <0.20) were evaluated in the multivariate model. We set $P < 0.05$ as the level of statistical significance. Data were analyzed using SPSS software version 11.0 for Windows (SPSS, Chicago, IL).

results

patient characteristics

A total of 228 patients were analyzed, of whom 87 (38.2%) were given CHOP and 141 (61.8%) were given RCHOP. The median SIL-2R was 1005.5 mg/dl (range 220–35 600), and high SIL-2R was observed in 114 (50.0%) patients: 40 of 87 (46.0%) in the CHOP group and 74 of 141 (52.5%) in the RCHOP group. There was no significant difference in the proportion of high SIL-2R patients between the two treatment groups. The characteristics of the patients are listed in Table 1. Patient and disease characteristics were well balanced between the groups.

survival analysis

With median follow-up periods of 30 months in the RCHOP group and 44 months in the CHOP group, EFS rates at 2 years were 78% and 65%, respectively ($P = 0.030$), and OS rates at 2 years were 89% and 81%, respectively ($P = 0.040$).

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Table 1. Patients' characteristics according to serum SIL-2R level for CHOP and RCHOP group

Characteristics	CHOP group			RCHOP group			*P*, all
	All	Low SIL2R	High SIL2R	All	Low SIL2R	High SIL2R	
No. of patients (%)	87(100)	47 (54)	40 (46)	141 (100)	67 (48)	74 (52)	
Sex, no. (%)							0.41
Male	50 (57)	27 (57)	23 (58)	72 (51)	27 (40)	45 (61)	
Female	37 (43)	20 (43)	17 (42)	69 (49)	40 (60)	29 (39)	
Age, no. (%)							0.52
≤60	24 (28)	13 (28)	11 (28)	45 (32)	29 (43)	16 (22)	
>60	63 (72)	34 (72)	29 (72)	96 (68)	38 (57)	58 (78)	
LDH, no. (%)							0.54
Normal	29 (32)	22 (35)	7 (17)	68 (48)	45 (67)	23 (31)	
High	58 (68)	25 (65)	33 (83)	73 (52)	22 (33)	51 (69)	
PS, no. (%)							0.81
0–1	77 (89)	44 (94)	33 (83)	127 (90)	66 (98)	61 (82)	
2–3	10 (11)	3 (6)	7 (17)	14 (10)	1 (2)	13 (18)	
Stage, no. (%)							0.73
I, II	55 (63)	40 (85)	15 (38)	93 (66)	57 (85)	36 (49)	
III, IV	32 (37)	7 (15)	25 (72)	48 (34)	10 (15)	38 (51)	
Extranodal sites, no. (%)							0.84
0, 1	67 (77)	43 (91)	24 (60)	106 (75)	63 (94)	43 (57)	
≤2	20 (23)	4 (9)	16 (40)	35 (25)	4 (6)	31 (43)	
IPI, no. (%)							0.86
L/L-I	60 (69)	40 (85)	20 (50)	100 (71)	63 (94)	37 (50)	
H/H-I	27 (31)	7 (15)	20 (50)	41 (29)	4 (6)	37 (50)	

SIL2R, soluble interleukin-2 receptor; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone; RCHOP, rituximab-combined CHOP; LDH, lactate dehydrogenase; PS, performance status; IPI, International Prognostic Index; L/L-I, low or low-intermediate; H/H-I, high or high-intermediate; high SIL-2R; SIL-2R >1000 U/ml, low SIL-2R; SIL-2R ≤1000 U/ml.

For CHOP therapy, the EFS and OS rates at 2 years were 82% and 93% for low SIL-2R and 43% and 65% for high SIL-2R, respectively. The differences in both the EFS and OS rates between the two SIL-2R levels were significant (both $P < 0.001$) (Figure 1A and B). In the RCHOP group, the EFS and OS rates at 2 years were 90% and 95% for low SIL-2R and 66% and 84% for high SIL-2R, respectively. The differences in both EFS and OS rates between the two SIL-2R levels were significant (EFS, $P < 0.001$; OS $P = 0.005$) (Figure 1C and D).

To study the impact of rituximab on the predictive value, we examined the clinical outcome according to treatment in the SIL-2R low and high groups. The patients with high SIL-2R who received RCHOP therapy had a significantly better OS at 2 years than patients treated with CHOP alone (84% versus 65%, $P = 0.020$). The EFS at 2 years was estimated to be 66% for the RCHOP group and 43% for the CHOP group ($P = 0.010$). For the patients with low SIL-2R, the influence of rituximab on OS and EFS was not significant (OS, 93% versus 95%, $P = 0.310$; EFS, 82% versus 90%, $P = 0.160$) (Table 2).

For comparison with this parameter, we analyzed the survival curves according to the IPI in both treatment groups. The EFS and OS rates at 2 years were 35% and 59% for high or high-intermediate IPI and 77% and 91% for low or low-intermediate IPI, respectively, in the CHOP group. The differences in both EFS and OS rates between the two IPI groups were significant (both $P < 0.001$). Similarly, the EFS and OS rates were 58% and 80% for high or high-intermediate IPI and 86% and 94% for low or low-intermediate IPI, respectively, in the RCHOP group. Again, the differences in the EFS and OS rates were significant ($P < 0.001$ and $P = 0.004$, respectively).

To estimate unbiased prognostic impacts, Cox univariate analysis showed that a high SIL-2R level, high PS, advanced stage, multiple extranodal sites and high or high-intermediate risk of IPI were associated with poor EFS and OS in both treatment groups (Table 3). In the second step, Cox multivariate analysis showed that only SIL-2R was significantly associated with a higher risk of event and that SIL-2R and PS were independently associated with poor OS in both treatment groups (Table 4). Finally, SIL-2R was a significant risk factor for EFS and a borderline risk factor for OS in both the CHOP and RCHOP groups ($P = 0.060$ and 0.070, respectively), whereas IPI was a significant risk factor for EFS and OS in the CHOP group and a borderline significant risk factor for EFS and OS ($P = 0.070$ and 0.080, respectively) in the RCHOP group (Table 5).

discussion

Although SIL-2R is easy to measure, its prognostic value has been underestimated due to its evaluation in smaller populations than those for other parameters, such as IPI [2]. The SIL-2R level was reported to be significantly high in highly aggressive lymphomas [6] and subsequently was recognized to reflect tumor burden and poor outcome [3–8]. However, these

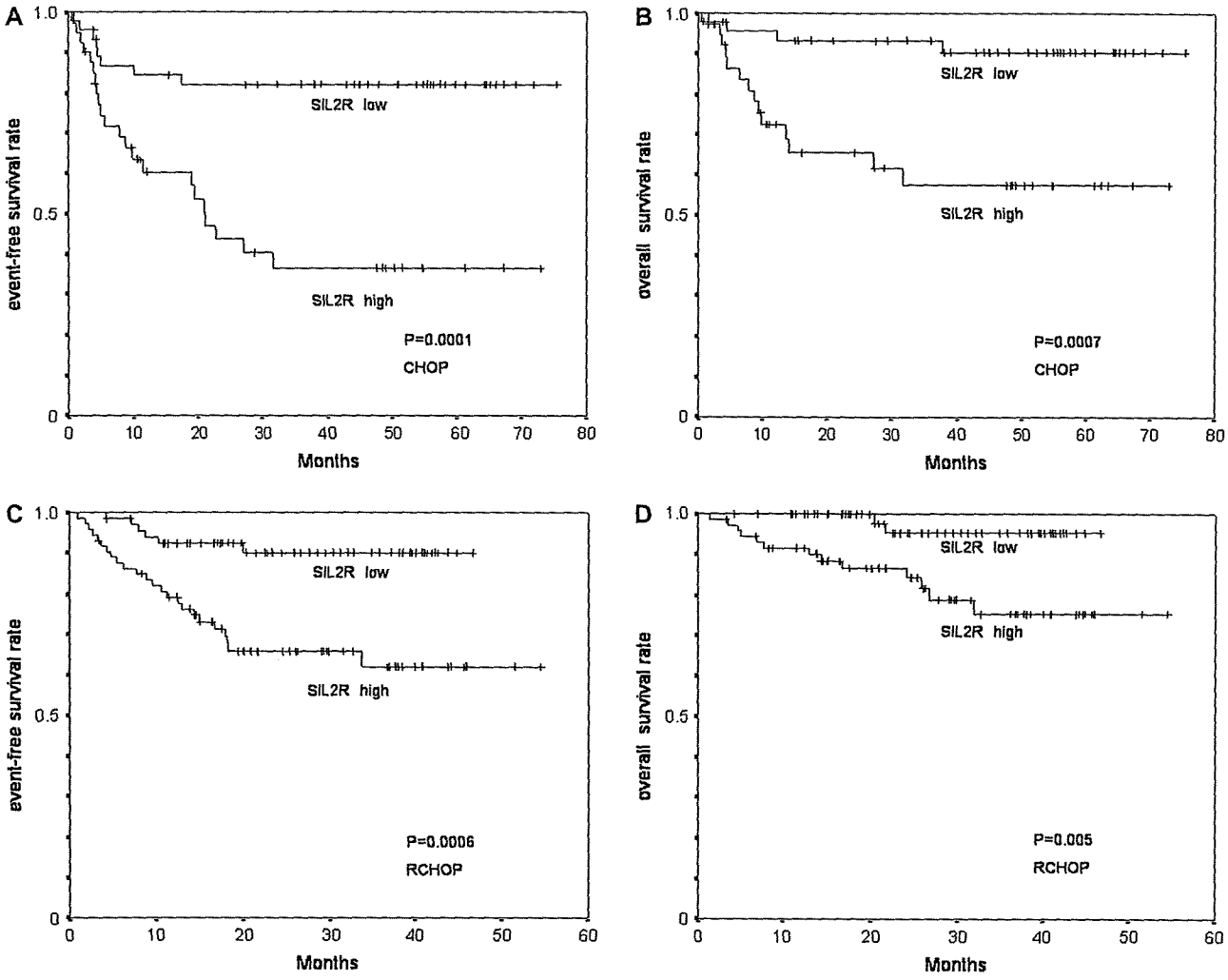

Figure 1. Event-free survival (EFS) and overall survival (OS) curves for diffuse large B-cell lymphoma patients treated with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) and rituximab-combined CHOP (RCHOP) in relation to soluble interleukin-2 receptor (SIL-2R). EFS (A) and OS (B) curves according to low ($n = 47$) versus high ($n = 40$) SIL-2R in the CHOP group. EFS (C) and OS (D) curves according to low ($n = 67$) versus high ($n = 74$) SIL-2R in the RCHOP group.

Table 2. Analysis of 2-year survival rate according to CHOP and RCHOP therapy in both SIL-2R groups

Clinical outcome	Low SIL-2R			High SIL-2R		
	CHOP	RCHOP	*P*	CHOP	RCHOP	*P*
2-year survival						
EFS (%)	82	90	0.160	43	66	0.0010
OS (%)	93	95	0.310	65	84	0.0020

CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone; RCHOP, rituximab-combined CHOP; SIL2R, soluble interleukin-2 receptor; EFS, event-free survival; OS, overall survival.

results were obtained in patients receiving chemotherapy, and the prognostic value of SIL-2R has not been assessed in rituximab-combined treatment.

In the present study, univariate analysis showed that SIL-2R retained its prognostic value in DLBCL patients treated with RCHOP, as well as in those receiving CHOP alone. Multivariate analysis also showed that SIL-2R was an independent significant prognostic factor after adjustment for IPI risk factors and independently associated with significantly decreased EFS and moderately decreased OS after adjustment by two-categorized IPI in both the CHOP and RCHOP groups. On the other hand, the clinical outcome of patients with high SIL-2R was significantly improved by addition of rituximab to the chemotherapy, in contrast to the lack of any difference in the patients with low SIL-2R. To our knowledge, this is the first report to demonstrate the prognostic value of SIL-2R in DLBCL patients treated with rituximab-combined chemotherapy.

Although the present study was not a randomized prospective one, and possibly biased by factors other than IPI and SIL-2R, the distribution of baseline characteristics, including IPI factors, was similar between the two treatment groups. On the other hand, the population employed in the present analysis had more limited disease and a favorable IPI score compared with those in previous studies of DLBCL [13–17]. This might account for the better outcome of our

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Table 3. The effects of clinical factors on EFS and OS in CHOP by univariate analysis using Cox proportional hazard model

	Variable	HR	95% CI	P value
CHOP				
EFS	SIL-2R			
	Low	1.00		
	High	4.30	1.94–9.74	<0.001
	Age			
	≤60	1.00		
	>60	1.48	0.64–3.46	0.36
	LDH			
	Normal	1.00		
	High	2.65	0.80–8.74	0.11
	PS			
	0–1	1.00		
	2–3	4.10	1.64–10.25	0.003
	Stage			
	I, II	1.00		
	III, IV	3.75	1.79–7.83	<0.001
	Extranodal sites			
	0–1	1.00		
	≤2	3.24	1.54–6.81	0.002
	IPI			
	L/L-I	1.00		
	H/H-I	3.97	1.91–8.25	<0.001
OS	SIL-2R			
	Low	1.00		
	High	5.64	1.84–17.23	0.002
	Age			
	≤60	1.00		
	>60	2.20	0.64–7.61	0.21
	LDH			
	Normal	1.00		
	High	4.71	0.63–35.41	0.13
	PS			
	0–1	1.00		
	2–3	7.18	2.44–21.13	<0.001
	Stage			
	I, II	1.00		
	III, IV	5.15	1.92–13.81	0.001
	Extranodal sites			
	0-1	1.00		
	≤2	4.24	1.64–10.98	0.003
	IPI			
	L/L-I	1.00		
	H/H-I	7.28	2.69–19.67	<0.001
RCHOP				
EFS	SIL-2R			
	Low	1.00		
	High	4.20	1.72–10.33	0.002
	Age			
	≤60	1.00		
	>60	1.38	0.61–3.11	0.44
	LDH			
	Normal	1.00		
	High	1.41	0.68–2.93	0.35
	PS			
	0–1	1.00		
	2–3	3.62	1.35–8.46	0.003
	Stage			
	I, II	1.00		
	III, IV	3.42	1.64–7.11	0.001
	Extranodal sites			
	0–1	1.00		
	≤2	3.43	1.67–7.03	<0.001
	IPI			
	L/L-I	1.00		
	H/H-I	3.40	1.82–6.25	<0.001
OS	SIL-2R			
	Low	1.00		
	High	6.42	1.45–28.45	0.01
	Age			
	≤60	1.00		
	>60	3.50	0.79–15.52	0.10
	LDH			
	Normal	1.00		
	High	1.69	0.58–4.97	0.34
	PS			
	0–1	1.00		
	2–3	5.97	2.03–17.54	0.001
	Stage			
	I, II	1.00		
	III, IV	2.46	0.89–6.79	0.08
	Extranodal sites			
	0–1	1.00		
	≤2	2.65	0.96–7.33	0.06
	IPI			
	L/L-I	1.00		
	H/H-I	4.03	1.43–11.34	0.08

EFS, event-free survival; OS, overall survival; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone; RCHOP, rituximab-combined CHOP; HR, hazard ratio; CI, confidential interval; SIL2R, soluble interleukin-2 receptor; LDH, lactate dehydrogenase; PS, performance status; IPI, International Prognostic Index; L/L-I, low or low-intermediate; H/H-I, high or high-intermediate.

patients than for those in previous reports such as that by Coiffier et al. [14] who observed 2-year survival rates of 70% and 57% in elderly patients treated with RCHOP and CHOP, respectively. Even with the excellent outcome we observed, however, the prognostic value of SIL-2R was significant and greater than that of other IPI risk factors. To allow our present results to be generalized to routine patient care, these findings should be validated in a variety of patient populations.

A number of prognostic markers have been identified in patients with DLBCL treated by chemotherapy alone [19–21], some of which have been reassessed and shown not to be associated with prognosis in patients receiving rituximab-combined chemotherapy [22–24]. BCL2 overexpression was reported to be associated with poorer survival in patients treated with CHOP-like regimens [19], but its prognostic value was not confirmed in patients receiving rituximab-combined chemotherapy in several studies, indicating that addition of rituximab overcomes the negative influence of BCL2

Table 4. Multivariate Cox proportional hazard regression analysis for SIL-2R and IPI risk factors in both treatment groups

	Variable	HR	95% CI	*P* value
CHOP				
EFS	SIL-2R			
	Low	1.00		
	High	2.74	1.05–7.14	0.04
	PS			
	0–1	1.00		
	2–3	2.12	0.73–6.14	0.17
	Stage			
	I, II	1.00		
	III, IV	1.82	0.65–5.09	0.25
	Extranodal sites			
	0–1	1.00		
	≤2	1.09	0.38–3.10	0.87
OS	SIL-2R			
	Low	1.00		
	High	3.53	1.03–12.95	0.05
	LDH			
	Normal	1.00		
	High	3.21	0.40–25.51	0.27
	PS			
	0–1	1.00		
	2–3	3.60	0.98–13.20	0.05
	Stage			
	I, II	1.00		
	III, IV	2.00	0.51–7.87	0.32
	Extranodal sites			
	0–1	1.00		
	≤2	0.86	0.22-0.83	0.83
RCHOP				
EFS	SIL-2R			
	Low	1.00		
	High	2.65	1.01–7.30	0.05
	PS			
	0–1	1.00		
	2–3	1.66	0.62–4.42	0.31
	Stage			
	I, II	1.00		
	III, IV	1.69	0.65–4.42	0.28
	Extranodal sites			
	0–1	1.00		
	≤2	1.36	0.50–3.67	0.55
OS	SIL-2R			
	Low	1.00		
	High	5.09	1.00–25.88	0.05
	Age			
	≤60	1.00		
	>60	2.45	0.54–11.17	0.24
	PS			
	0–1	1.00		
	2–3	4.49	1.15–17.45	0.03
	Stage			
	I, II	1.00		
	III, IV	1.02	0.23–4.45	0.98
	Extranodal sites			
	0–1	1.00		
	≤2	0.70	0.15–3.39	0.66

SIL2R, soluble interleukin-2 receptor; IPI, International Prognostic Index; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone; HR, hazard ratio; CI, confidential interval; EFS, event-free survival; PS, performance status; OS, overall survival; LDH, lactate dehydrogenase; RCHOP, rituximab-combined CHOP.

Table 5. Multivariate Cox proportional hazard analysis for SIL-2R and categorized IPI in both treatment groups

	Variable	HR	95% CI	*P* value
CHOP				
EFS	SIL-2R			
	Low	1.00		
	High	2.98	1.22–7.29	0.01
	IPI			
	L/L-I	1.00		
	H/H-I	2.47	1.11–5.47	0.02
OS	SIL-2R			
	Low	1.00		
	High	3.12	0.93–10.41	0.06
	IPI			
	L/L-I	1.00		
	H/H-I	4.66	1.60–13.58	0.005
RCHOP				
EFS	SIL-2R			
	Low	1.00		
	High	3.00	1.12–8.07	0.02
	IPI			
	L/L-I	1.00		
	H/H-I	2.06	0.93–4.57	0.07
OS	SIL-2R			
	Low	1.00		
	High	4.30	0.85–21.91	0.07
	IPI			
	L/L-I	1.00		
	H/H-I	4.16	0.80–6.69	0.08

SIL2R, soluble interleukin-2 receptor; IPI, International Prognostic Index; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone; HR, hazard ratio; CI, confidential interval; EFS, event-free survival; OS, overall survival; LDH, lactate dehydrogenase; RCHOP, rituximab-combined CHOP; L/L-I, low or low-intermediate; H/H-I, high or high-intermediate.

overexpression [24]. BCL6, a marker of germinal center derivation, has been identified as an indicator of favorable outcome in DLBCL [20], although outcome in patients receiving immunochemotherapy was reported to be uninfluenced by BCL6 status [22]. Similarly, no correlation between immunohistochemically defined GC phenotype and survival rate was observed in patients receiving immunochemotherapy [21], in contrast to previous findings of inferior outcomes in non-GC patients relative to GC patients in the prerituximab era [23]. Up to now, no marker other than IPI has been found to be of prognostic relevance since the clinical introduction of rituximab.

The mechanism by which rituximab added to chemotherapy improves outcome in relation to biological features has been evaluated in several studies. They showed that rituximab may suppress the constitutively active nuclear factor-kappa B pathway in non-GC phenotype DLBCL or downregulate Bcl-2-related antiapoptotic proteins, thereby increasing the sensitivity of lymphoma cells to chemotherapy [27–29]. These effects of rituximab may reduce the prognostic significance of the non-GC phenotype and BCL2. Although the mechanism by which SIL-2R retains its prognostic value after addition of rituximab to chemotherapy is unknown, SIL-2R may directly represent the tumor burden [7, 8].

To date, several studies including the present one have demonstrated that IPI score remains predictive in the rituximab era, in contrast to biomarkers [18, 22, 23]. In the present study, the IPI system identified only two risk groups instead of four among our patients—a low and low-intermediate group and a high and high-intermediate group—as reported in previous studies [22, 23].

The addition of rituximab to chemotherapy has improved the outcome of patients. We and others have shown that OS now exceeds 50% even in the groups with unfavorable indicators [14–18, 22, 23], although some patients still have a very poor outcome. Therefore, other predictive factors must be characterized in order to identify patients who should receive alternative initial therapy. A number of molecular prognostic markers have already been identified in patients with DLBCL [30]. These markers now need to be reevaluated in the rituximab era to identify patients with unfavorable prognostic factors and to devise adequate treatment strategies.

In conclusion, we have demonstrated that a high serum SIL-2R level is an indicator of poor prognosis in DLBCL patients receiving rituximab combination chemotherapy. To accurately confirm whether serum SIL-2R influences the outcome of patients receiving rituximab combination chemotherapy, prospective investigation with long-term follow-up will be required.

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Human papillomavirus 6 seropositivity is associated with risk of head and neck squamous cell carcinoma, independent of tobacco and alcohol use

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Background: The risk of head and neck squamous cell carcinoma (HNSCC) associated with common human papillomavirus types has not been well defined.

Methods: We conducted a case–control study of 1034 individuals (486 incident cases diagnosed with HNSCC and 548 population-based controls matched to cases by age, gender, and town of residence) in Greater Boston, MA. Sera were tested for antibodies to human papillomavirus (HPV)6, HPV11, HPV16, and HPV18 L1.

Results: HPV6 antibodies were associated with an increased risk of pharyngeal cancer [odds ratio (OR) = 1.6, 1.0–2.5], controlling for smoking, drinking, and HPV16 seropositivity. In HPV16-seronegative subjects, high HPV6 titer was associated with an increased risk of pharyngeal cancer (OR = 2.3, 1.1–4.8) and oral cancer (OR = 1.9, 1.0–3.6), suggesting that the cancer risk associated with HPV6 is independent of HPV16. There was no association between smoking and alcohol use and HPV6 serostatus. Further, the risk of pharyngeal cancer associated with heavy smoking was different among HPV6-seronegative (OR 3.1, 2.0–4.8) and HPV6-seropositive subjects (OR = 1.6, 0.7–3.5), while heavy drinking also appears to confer differing risk among HPV6-negative (OR 2.3, 1.5–3.7) and -positive subjects (OR = 1.3, 0.6–2.9).

Conclusions: There may be interactions between positive serology and drinking and smoking, suggesting that the pathogenesis of human papillomavirus in HNSCC involves complex interactions with tobacco and alcohol exposure.

Key words: epidemiology, head and neck squamous cell carcinoma, human papillomavirus, risk factors, serology

introduction

Heavy alcohol and tobacco use are thought to account for the majority of the estimated 40 000 head and neck squamous cell carcinoma (HNSCC) cases diagnosed per year in the United States [1], with the well-described synergistic interaction between these exposures responsible for induction of much of this disease. Recent studies have shown that infection with high-risk human papillomavirus (HPV) is also a risk factor for HNSCC [2, 3]. Approximately 25% of HNSCC cases have detectable HPV DNA in tumor tissue [2]. Positive HPV16 L1 serology reflects exposure to HPV16 virus and is also associated with increased risk for HNSCC [4]. The majority of HPV DNA-positive HNSCC is positive for HPV16 [2, 4–7], and large studies of HPV serology have focused on detecting HPV16 antibodies [7–10]. Other HPV types, including high-risk type HPV18, however, also have been detected in HNSCC [2, 3, 11], but few studies of HPV serology in HNSCC have examined the seroprevalence of multiple types.

Unlike cervical cancer, where HPV is the necessary cause of disease [12, 13], the contribution of HPV to the development of HNSCC may be more complex, as this disease is primarily associated with other carcinogenic exposures, including alcohol and tobacco. Data suggest that the neoplastic transformation that occurs in the upper aerodigestive tract as a result of the carcinogenic action of alcohol and tobacco may be intensified or somehow altered by HPV infection [12, 14].

Case–control studies that have evaluated cumulative exposure to HPV16 by measuring serum anti-HPV16 L1 antibodies have been inconsistent with regard to the interaction between smoking and HPV16 serology in predicting risk for HNSCC [7–9]. Studies examining the role of HPV infection in HNSCC have found that viral presence (a marker of ongoing infection) is inversely associated with heavy alcohol and tobacco exposure [2, 6, 15]. Thus, exposure to alcohol and

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

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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%) or transmembrane 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

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Translational Relevance

Rituximab is commonly incorporated into CD20-positive B-cell lymphoma therapy to improve response and prognosis. However, with increasing use, resistance to rituximab is a continuing concern. Although some mechanisms have been explained for resistance to rituximab, CD20 C-terminal mutation was found as one of the mechanism for the first time. In this study, two useful applications will be of concern in the field of medicine of malignant lymphoma. First, because the CD20 C-terminal mutation was detected in only patients with disease progression, a more sensitive assay could be developed to detect CD20 mutations at initial diagnosis. This will be able to predict whether the patients with the CD20 mutation may show relapsed/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 calicheamycin. For those reasons, this work will be applied to future important practice of the field of malignant lymphoma.

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 lymphoma cells, and these cells showed loss of CD20 expression. In this article, we analyze the relationships between CD20 mutation, CD20 expression, and relapse after rituximab therapy in 50 patients with lymphoma, including the original index case.

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 do 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/m^2 weekly for 8 cycles and cyclophosphamide 750 mg/m^2, doxorubicin 50 mg/m^2, vincristine 1.4 mg/m^2, and prednisolone 60 mg/m^2) therapy. Three of them underwent radiotherapy to a total dose of 35 to 45 Gy after R-CHOP, and one of them received radiotherapy before R-CHOP. One patient received a therapy of rituximab 375 mg/m^2 weekly for 8 cycles and cyclophosphamide 750 mg/m^2, vincristine 1.4 mg/m^2, and prednisolone 60 mg/m^2. Five patients received rituximab monotherapy (375 mg/m^2 weekly for 8 cycles). For one patient, rituximab-VP-16 was given as rituximab 375 mg/m^2 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 *Bam*HI and *Sal*I 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/Amplimax method with the following temperature cycling parameters: 95°C for 30 s, 58°C for 30 s, 68°C for 1 min for 25 cycles, and a final extension at 68°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′-CCTTTCTCAGAACTCAGC AGTAGGCCTTGC-3′; reverse primer for exon 3, 5′-ACTGACTTACCCCCAAAGTCTTAGATTCCC-3′; forward primer for exon 4, 5′-CTCTCCCCAGGCTGTCCAGATTATGAATGG-3′; reverse primer for exon 4, 5′-TTTTACTCACCATAATGCCTCCCCAGAGAG-3′; forward primer for exon 5, 5′-CTCCTCTATCTCCTGTCTTGCCCACCCCCT-3′; reverse primer for exon 5, 5′-AAAAAATAGGTACTTCTCTGACATGTGGGA-3′; forward primer for exon 6, 5′-CATTTCAGGTCAAAGGAAAAATGAT-3′; reverse primer for exon 6, 5′-ACTTACCAAGAACACTTACCAAGAA-3′; forward primer for exon 7, 5′-TGTTTTTCAGGGCATTTTGTCAGTGATGCT-3′; reverse primer for exon 7, 5′-ACTACTACTTACAGATTTGGGTCTGGAGCA-3′; forward primer for exon 8, 5′-TTTCTGTTTTAGAACATAGTTCTCCTGTCA-3′; and reverse primer for exon 8, 5′-CAGAAAACAGAAGAAATCACTTAAGGAGAG-3′.

Table 1. Patient characteristics

Histology	Treatment	*n*	Analysis of PD sample
MALT	R-CHOP	11	1
	R	3	1
	R-VP16	1	
	R-CHOP → RTx	1	
FL	R-CHOP	5	2
	R	1	
	R-CHOP → RTx	1	
DLBCL	R-CHOP	21	2
	RTx → R-CHOP	1	1
CLL/SLL	R-CHOP	2	1
	R	1	
Lymphoplasmacytic	R-COP	1	
MCL	CHOP+ RTx →R	1	1

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′-CGCGGATCCGCGATGACAACACCCAGA-3′; reverse primer for full length, 5′-TCCCCCGGGGGATTAAGGAGAGCTGTC-3′; forward primer for exons 3 and 4, 5′-ATGACAACACC CAGAAATTCAGTAAATGGG-3′; reverse primer for exons 3 and 4, 5′-CATAATGCCTCCCCAGAGAGGG-TACCACAC-3′; forward primer for exons 5 and 6, 5′-TATATTATTTCCG-GATCACTCCTGGCAGCA-3′; reverse primer for exons 5 and 6, 5′-CCAAGAACAGAGATTGTATGCTGTAACAGT-3′; forward primer for exons 7 and 8, 3′-GCATTTTGTCAGTGATGCTGATCTTTGCCT-5′; and reverse primer for exons 7 and 8, 5′-TTAAGGAGAGCT GTCATTTTC-TATTGGTG A-3′. The following pair of primers for glyceraldehyde-3-phosphate dehydrogenase was used as a housekeeping gene control: forward primer, 5′-CCTTCATTGACCTCAACTAC-3′, and reverse primer, 5′-AGTGATGGCATGGACTGTGGT-3′.

Direct sequence analysis of genomic DNA and PCR product was done by ABI PRISM 3100 (Invitrogen) 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 μCi of ^{35}S-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 (Daiichikagaku), 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 diaminobenzidene (DAB+, DAKO). The sections were counterstained with hematoxylin; all staining procedures were done in a DAKO Autostainer.

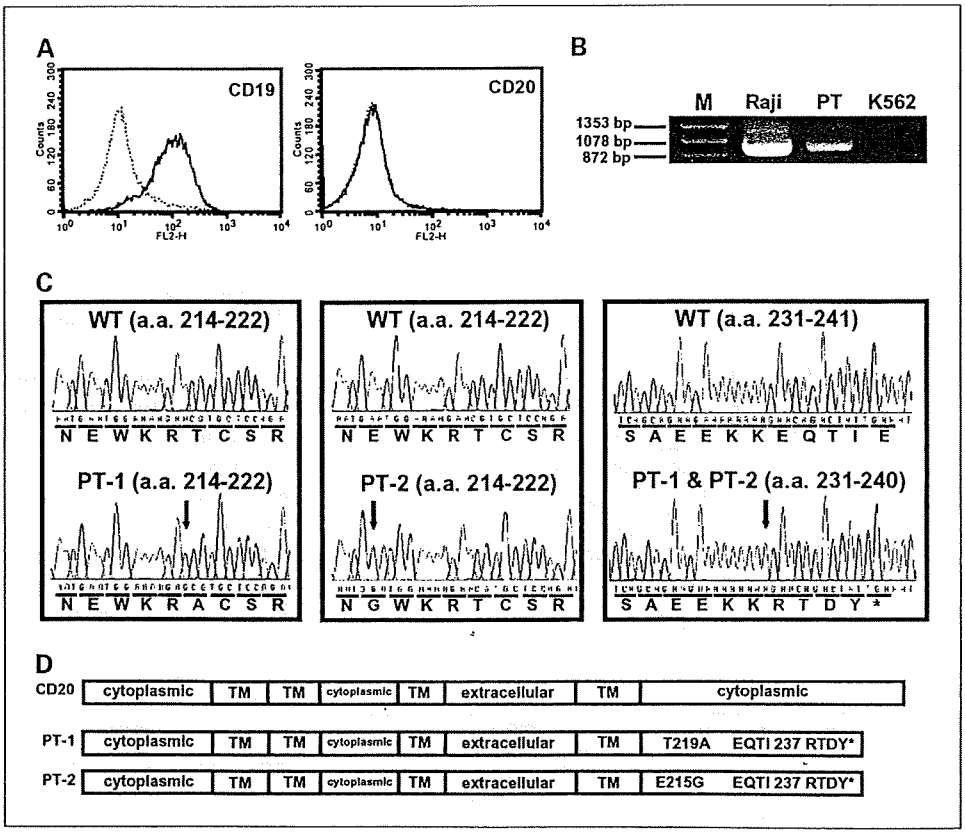

Fig. 1. CD20 expression and mutation analysis in CD19-positive lymphoma cells from a patient with posterior mediastinal lymphoma. *A*, flow cytometry for CD19 and CD20 cells. *B*, RNA analysis. RT-PCR using CD20 primers. Total RNAs from Raji and K562 cells were used as positive and negative controls, 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-I240) were also changed at the C-terminus (R237-Y240). PT, patient.

Table 2. Mutations found in 11 patients

Groups	Mutated domains	Amino acid sequence from mutation	Classification	Therapy	Biopsy after PD
Group 1					
C-terminal deletion (truncation) CD-1	C-terminal cytoplasmic	I211S	FL	R-CHOP	Yes
CD-2	C-terminal cytoplasmic	EQT123 RTDY	DLBCL	R-CHOP	No
CD-3*	C-terminal cytoplasmic	T219A: EQT123RTDY	DLBCL*	RTx → R-CHOP	Yes*
CD-4*	C-terminal cytoplasmic	E215G: EQT123RTDY	DLBCL*	RTx → R-CHOP	Yes*
CD-5	Second transmembrane fused to C-terminal cytoplasmic	SLLAATEKNSRKCLVKGKMIMNSLSLFAAIS-GMILSIMDIL fused to ITPGSNGEKLQEV-FGQRKNDNEFIEPLCCHFWNDSFNHGHT	MCL	CHOP + RTx → R	Yes
Group 2					
Extracellular	Extracellular	T180A	DLBCL	R-CHOP	No
Group 3					
Transmembrane	Third transmembrane	F125L	CLL/SLL	R	No
Group 4					
Early termination	N-terminal cytoplasmic	MYIHVLKLSHHFMSTVH	MALT	R-CHOP	No
	N-terminal cytoplasmic	MGLSRQSQ	DLBCL	R-CHOP	No
	N-terminal cytoplasmic	MGLSRQSQ	DLBCL	R-CHOP	No
	N-terminal cytoplasmic	MTHPEIQ	MALT	R-CHOP	No
	N-terminal cytoplasmic	MTHPEIQ	DLBCL	R-CHOP	No

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

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.

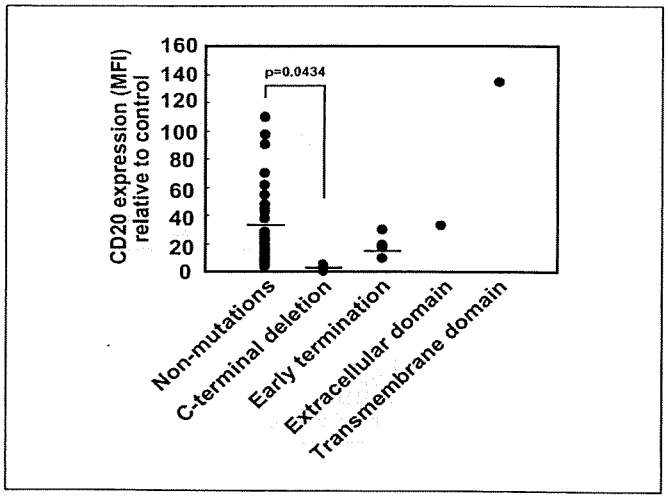

Fig. 2. Relationship between mutations and CD20 expression. Mean fluorescence intensity of CD20 relative to the control was assessed in CD19-positive cells from clinical samples by flow cytometric analysis. Fifty cases were classified as nonmutations or as mutations and grouped according to the domain affected (C-terminal deletion, extracellular domain, early termination, and transmembrane domain). MFI, mean fluorescence intensity.

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.

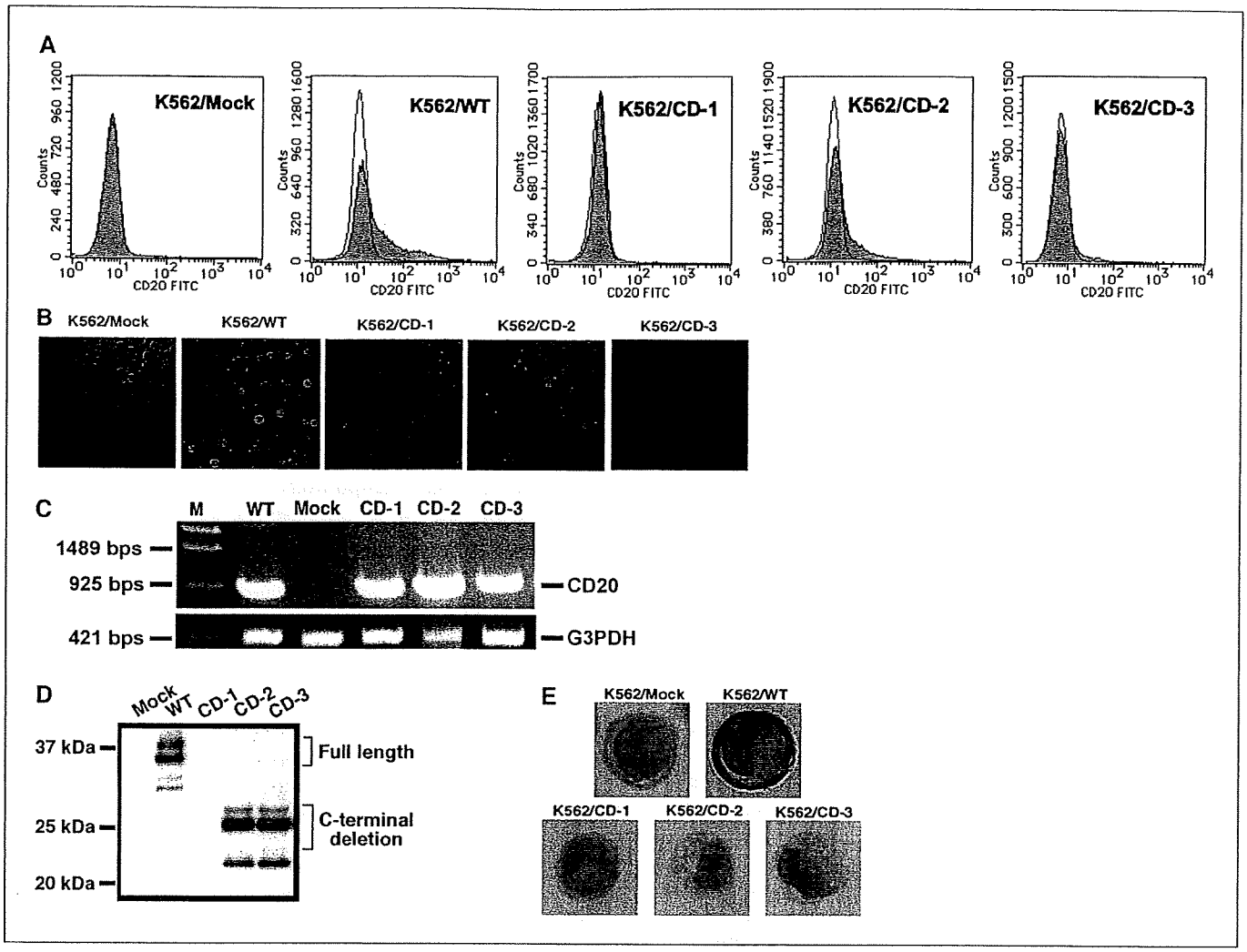

Fig. 3. CD20 expression in mutants CD-1, CD-2, and CD-3. Wild-type and mutant sequences were stably transfected into K562 cells (K562/mock, K562/WT, K562/CD-1, K562/CD-2, and K562/CD-3); after which, flow cytometric analysis was done with Alexa Fluor 488–labeled rituximab, and confocal laser scanning microscopy with Alexa Fluor 488–labeled rituximab was used for imaging analysis (FV1000, Olympus). *A*, flow cytometry results. *B*, confocal laser scanning microscopy results. *C*, RT-PCR results using total RNA. The PCR product of glyceraldehyde-3-phosphate dehydrogenase was loaded as a housekeeping gene control. *D*, Western blotting results using anti–CD20 N-terminus antibody. *E*, immunohistochemistry results. Cells were stained for CD20 expression with anti–CD20 N-terminus antibody.

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-HFWNDSFNHGHT 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, 51.82; 95% CI, 1-133.3), and the specimens collected upon progression of disease (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, 36.4; 95% CI, 25.7-47.1). 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 cells 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.

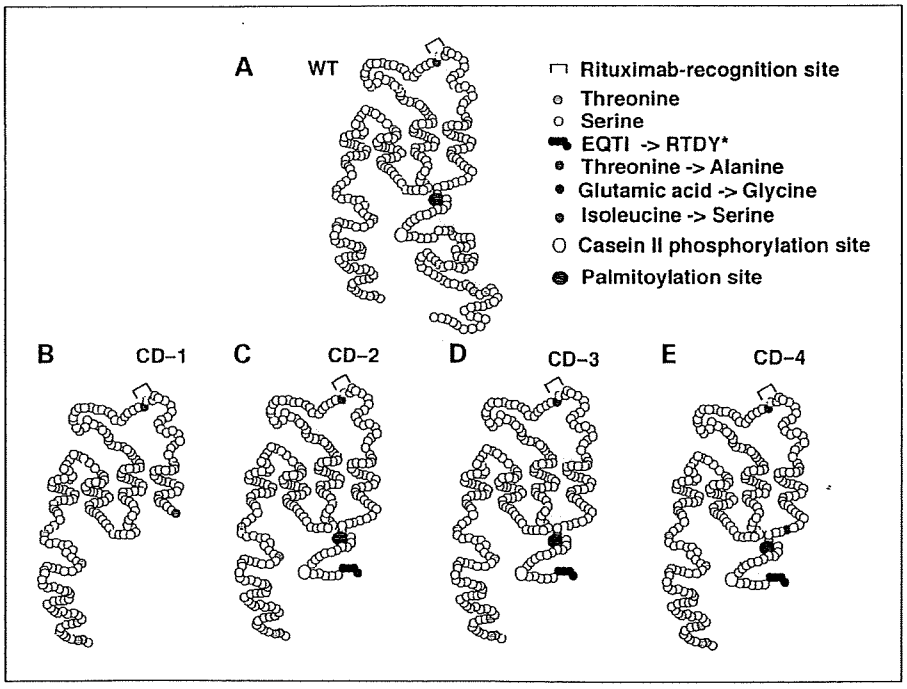

Fig. 4. The structures of wild-type and mutant CD20. Wild-type CD20 is composed of 297 amino acids (*A*), and casein kinase 2 and calcium/calmodulin-dependent protein kinase 2 phosphorylation sites are expected in its C-terminal cytoplasmic domain. C-terminal deletion mutants are shown as CD-1 (*B*), 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/calmodulin-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 Motif Scan 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 cause induced 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 immunochemotherapy, 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.

Disclosure of Potential Conflicts of Interest

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An Imaging-Based Rapid Evaluation Method for Complement-Dependent Cytotoxicity Discriminated Clinical Response to Rituximab-Containing Chemotherapy

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Abstract

Purpose: Rituximab has greatly improved the efficacy of chemotherapy regimens for CD20-positive non-Hodgkin's lymphoma. However, although several mechanisms of action of rituximab have been identified, the exact therapeutic functions of these mechanisms remains to be clarified. In addition, there is no established prognostic marker to predict an individual response. This study verified the validity of *ex vivo* complement-dependent cytotoxicity (CDC) susceptibility as a predictor of pathologic tumor regression in patients undergoing rituximab-containing chemotherapy and examined whether CDC contributes to the mechanism of action of rituximab.

Experimental Design: A rapid assay system was established to evaluate the tumoricidal activity of rituximab using a living cell–imaging technique. We analyzed lymph node biopsies obtained from 234 patients with suspected lymphomas and estimated the association between CDC susceptibility and the response to rituximab-containing chemotherapy in diffuse large B-cell lymphoma and follicular lymphoma.

Results: This study revealed that CDC susceptibility of lymphoma cells freshly obtained from patients was strongly associated with response to rituximab-containing chemotherapy in both diffuse large B-cell lymphoma and follicular lymphoma. This correlation was not apparent in cases that received chemotherapy without rituximab.

Conclusions: The system that we have established allows a successful assessment of rituximab-induced CDC and can distinguish cases refractory to rituximab-containing chemotherapy. The association between CDC susceptibility and therapy response suggests that CDC is pivotal in the ability of chemotherapy including rituximab to induce remission.

Although rituximab can be combined with chemotherapies used in the treatment of non-Hodgkin's lymphoma, efficacy varies from patient to patient. In addition, no prognostic marker to predict individual response has been established to date. Several mechanisms of action have been proposed and tested *in vitro*, mainly in tumor cell lines (1–3). Through its human IgG_1 Fc domain, rituximab can activate cellular effectors for antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis and can recruit serum proteins for complement-dependent cytotoxicity (CDC; ref. 4). Moreover, cross-linking of CD20 molecules on tumor cell lines has been reported to trigger apoptosis, as well as having an antiproliferative effect on some, but not all, cell lines (5, 6). Despite these insights, the mechanisms mediating tumor cell eradication *in vivo* are not well understood. Recently, analyses of FcγRIIIa polymorphisms have clearly shown that ADCC is one of the critical effector functions responsible for the clinical efficacy of therapeutic antibodies (7–9). The FcγRIIIa gene (*FCGR3A*) displays an allelic polymorphism that generates molecules containing either a phenylalanine (F) or a valine (V) at amino acid position 158, which is critical in mediating ADCC. A greater clinical response in patients with the FcγRIIIa allotype (FcgRIIIa-158V), which has a high affinity for human IgG_1, has been observed compared with results obtained from patients with the low-affinity allotype (FcγRIIIa-158F; ref. 10). These reports show the importance of ADCC in clinical outcomes. On the other hand, there are few reports that indicate a contribution to clinical effect in

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