Table 2. Patient Characteristics of Studies of Positron Emission Tomography for Interim Response Assessment of Malignant Lymphoma

Study	Year	No. of Participants Included	Clinical Staging*	Staging Before Therapy (No.)	Standard Prognostic Scores (No.)	Therapy	Use of Rituximab (%)
Advanced-stage HL			Inclusion criteria of advanced-stage		International Prognostic Scores		
Friedberg et al[33]	2004	22	IIB-IVB, any stage with bulky disease		NR	ABVD × 6 or MOPP/ABVD × 6 ± radiotherapy	—
Hutchings et al[37]	2005	28	IIB-IVB, any stage with bulky disease		NR	ABVD × 6 to 8 ± radiotherapy	—
Gallamini et al[30]	2006	108	IIB-IVB, IIA with adverse prognostic factors†		0 pts: 28, 1 pt: 34, 2 pts: 29, 3 pts: 10, 4 pts: 3, ≥ 5 pts: 4	ABVD × 6 or COPP/EBV/CAD × 6 ± radiotherapy	—
Hutchings et al[13]	2006	46	IIB-IVB		Median 3 pts	ABVD × 6 to 8 or comparable anthracycline-containing regimen ± radiotherapy	—
Kostakoglu et al[32]	2006	10	III-IV, any stage with bulky disease‡		0 pts: 3, 1 pt: 2, 2 pts: 4, 4 pts: 1	ABVD × 6	—
Zinzani et al [14]	2006	40	IIB-IVB		NR	ABVD × 6	—
Gallamini et al[29]	2007	106	IIB-IVB, IIA with adverse prognostic factors†		0 pts: 38, 1 pt: 70, 2 pts: 87, 3 pts: 42, 4 pts: 13, ≥ 5 pts: 10§	ABVD × 6, ABVD-like regimen × 6, or COPP/EBV/CAD × 6 ± radiotherapy	—
DLBCL					International Prognostic Indexes		
Spaepen et al[34]	2002	47		IA: 1, IIA: 15, IIB: 6, IIIA: 14, IIIB: 2, IVA: 14, IVB: 20§	L: 26, L-I: 22, H-I: 17, H: 17§	CHOP × 8, biweekly CHOP × 6, CHVmPBV × 8, or COP/COPADM/CYM × 6‖	0
Haioun et al[31]	2005	83		I-II: 8, III-IV: 82§	L: 14, L-I: 23, H-I: 30, H: 23§	(R-)CHOP × 8, R-ACVBP × 4¶, or ACVBP × 4 or ACE × 4#	45
Mikhaeel et al[12]	2005	57		I: 21, II: 14, III: 9, IV: 13	NR	(R-)CHOP × 6 or PMitCEBO × 6**	16
Fruchart et al[35]	2006	35		I-II: 13, III-IV: 27§	L: 13, L-I: 2, H-I or H: 15§	(R-)CHOP × 8 or (R-)ACVBP × 4††	74
Kostakoglu et al[32]	2006	24		I: 2, II: 11, III: 10, IV: 1	L: 16, L-I: 8	R-CHOP × 6 to 8	100
Querellou et al[38]	2006	21		I: 3, II: 2, III: 4, IV: 15§	L: 8, L-I: 5, H-I: 6, H: 5§	(R-)CHOP × 8, R-COP × 6, or (R-)CEEP × 4‡‡	90
Ng et al[36]	2007	44		I: 16, II: 9, III: 5, IV: 14	L: 17, L-I: 9, H-I: 12, H: 1, NA: 5	(R-)CHOP or CHOP-like regimen × 6 to 8, (R-)Hyper-CVAD × 8, or biweekly (R-) CHOP × 6 ± radiotherapy§§	40

Abbreviations: ABVD, doxorubicin, bleomycin, vinblastine, dacarbazine; ACE, doxorubicin, cyclophosphamide, etoposide; ACVBP, doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone; pts, patients; CAD, lomustine, doxorubicin, vindesine; CEEP, cyclophosphamide, epirubicin, vindesine, prednisone; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; CHVmPBV, cyclophosphamide, doxorubicin, teniposide, prednisone, bleomycin, vincristine; COP, cyclophosphamide, vincristine, prednisone; COPADM, cyclophosphamide, vincristine, prednisolone, doxorubicin, high-dose methotrexate; COPP, cyclophosphamide, vincristine, procarbazine, prednisone; CVAD, cyclophosphamide, vincristine, doxorubicin, dexamethasone; CYM, cytrabine, high-dose methotrexate; DLBCL, diffuse large B-cell lymphoma; EBV, epirubicin, bleomycin, vinblastine; H, high risk; H-I, high-intermediate risk; HL, Hodgkin's lymphoma; L, low risk; L-I, low-intermediate risk; MOPP, nitrogen mustard, vincristine, procarbazine, prednizone; NR, not reported; PMitCEBO, cyclophosphamide, mitoxantrone, etoposide, prednisolone, vincristine, bleomycin; R, rituximab.

*According to the Ann Arbor staging system.

†> 3 nodal cites, subdiaphragmatic involvement, bulky disease, erythrocyte sedimentation rate > 40 mm/hour.

‡Selected post hoc because of no information on B symptoms.

§Abstracted from total participants of original report, not exclusively for relevant patient population.

‖Some underwent high-dose chemotherapy followed by autologous stem-cell transplantation as consolidation therapy.

¶All received an eight-cycle biweekly consolidation therapy consisting high-dose methotrexate, etoposide, ifosfamide, and cytarabine after the ACVBP regimen.

#All underwent high-dose chemotherapy followed by autologous stem-cell transplantation with or without rituximab maintenance therapy.

**A portion of patients (n = 16) with limited-stage disease underwent 2 to 4 cycles of (R-)CHOP followed by involved field radiation therapy instead of full course (R-)CHOP.

††Patents with one age-adjusted international prognostic risk factor received an eight-cycle consolidation therapy, and patients with two or three factors underwent high-dose chemotherapy followed by autologous stem-cell transplantation.

‡‡All underwent high-dose chemotherapy followed by autologous stem-cell transplantation.

§§A portion of patients (n = 13) with limited-stage disease underwent 2 to 4 cycles of (R-)CHOP or similar regimens followed by involved field radiation therapy instead of full-cycle chemotherapy.

PET at varied timing ranging from the first to fifth cycle.[36] We contacted the investigators for individual patient data, and excluded one patient who underwent PET at the fifth cycle. We found one study[14] through hand searching of the reference lists. As a result, we included 13 studies: eight studies[13,14,29,30,32-35] that met all eligibility criteria and five studies[12,31,36-38] with unpublished data available through contacting the authors (Table 1).[12-14,29-36,38]

Study Characteristics

Thirteen included studies had 360 advanced-stage HL patients and 311 DLBCL patients (Table 1). Eight reports were prospective single- or multi-institutional studies enrolling adults or adolescents. Only one study evaluated both adults and children.[34] Most of the patients in the HL studies underwent PET after receiving two cycles of first-line chemotherapy, while the number of cycles before the PET scan varied in DLBCL studies. In three DLBCL studies, 25% to 52% of included patients underwent PET after the fourth cycle.[34,36,38] One study evaluated PET after one cycle.[32] In general, participants underwent PET during the second week of intended chemotherapy cycle for biweekly chemotherapies (eg, doxorubicin, bleomycin, vinblastine, dacarbazine [ABVD] or (R-) doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone [ACVBP]) and during the third week for triweekly regimens (eg, (R-) cyclophosphamide, doxorubicin, vincristine, prednisone [CHOP]). Four studies performed CT for a portion of patients at the same timing as interim PET but they did not perform direct comparison between the two tests.[13,30,36,38]

For advanced-stage HL studies, fewer than 10% of included patients had unfavorable risk by standard prognostic tool (IPS > 3 points; Table 2). Progression or relapse rates were between 20% and 30% except for one study of 50%.[32] All studies adopted currently available standard first-line chemotherapy: six to eight cycles of ABVD or comparable regimens with or without radiotherapy. For DLBCL studies, the percentage of patients with unfavorable prognosis (high-intermediate to high risk by IPI) ranged from 0% to 59%, with progression or relapse rates of 27% to 47%. Full course (R-) CHOP and (R-) ACVBP were the two most widely adopted regimens. Two studies employed abbreviated course of (R-) CHOP or comparable regimens followed by involved-field radiation for patients with limited-stage disease.[12,36] No patients received rituximab in one study.[34] In four studies, some patients received consolidation auto-transplant after induction chemotherapy.[31,34,35,38]

Concerning imaging techniques and technologies, included studies generally followed guidelines by the Society of Nuclear Medicine (Table 3). One study exclusively adopted combined PET/CT scanner.[38] In five studies, some patients underwent combined PET/CT while the others were evaluated with stand-alone dedicated PET scanner.[13,29,30,32,36] All but one study[34] adopted attenuation correction for image reconstruction.

In general, multiple experienced nuclear medicine physicians interpreted PET results with pretherapy baseline scan as reference. All studies adopted qualitative positive and negative diagnostic criteria with various definitions (online-only Appendix Table A3). Only two studies clearly reported the referential backgrounds to define positive lesion. Five studies defined MRU criterion,[12-14,29,37] which was eventually reported as negative in three studies.[13,14,29] No study reported between-observer variability.

Quality Assessment of Published Studies

Only two studies[13,35] reported all items of the QUADAS tool (online-only Appendix Table A4). Reporting was especially limited in

Table 3. Technical Specification of PET for Interim Response Assessment of Malignant Lymphoma

Study	Year	Preparation: Measurement of Blood Glucose	Procedure: Type of PET Scanner	Procedure: Time of Scan After Injection (minutes)	Procedure: Attenuation Correction	Procedure: Image Reconstruction Method	Procedure: Administered Activity (MBq)
Advanced-stage HL + DLBCL							
Kostakoglu et al[32]	2006	Yes	PET-CT or dedicated	60	Yes	OSEM	370-444
Advanced-stage HL							
Friedberg et al[33]	2004	Yes	Dedicated	50	Yes	OSEM	370
Hutchings et al[37]	2005	Yes	Dedicated	60	Yes	NR	350
Gallamini et al[30]	2006	Yes	PET-CT or dedicated	60	Yes	OSEM or RAMLA	370/70, 259/70, 2*
Hutchings et al[13]	2006	NR	PET-CT or dedicated	45-90	Yes	OSEM	400
Zinzani et al[14]	2006	NR	Dedicated	70-90	Yes	NR	6†
Gallamini et al[29]	2007	Yes	PET-CT or dedicated	60	Yes	OSEM or RAMLA	370/70, 259/70, 2*
DLBCL							
Spaepen et al[34]	2002	Yes	Dedicated	60	No	OSEM	370-555
Haioun et al[31]	2005	Yes	Dedicated	60	Yes	OSEM	2†
Mikhaeel et al[12]	2005	NR	Dedicated	60	Yes	NR	350
Fruchart et al[35]	2006	NR	Dedicated	60	Yes	OSEM	2.5†
Querellou et al[38]	2006	Yes	PET-CT	73 ± 15‡	Yes	OSEM	5.0-7.6†
Ng et al[36]	2007	Yes	PET-CT or dedicated	60-70	Yes	OSEM	5†

Abbreviations: CT, computed tomography; DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin's lymphoma; NR, not reported; OSEM, ordered subsets expectation maximization; PET, positron emission tomography; RAMLA, row-action maximum likelihood algorithm; SUV, standard uptake value.

*Three hundred seventy MBq/70 kg at the centers that used a GE scanner, 259 MBq/70 kg at the centers that used a Philips scanner, and 2 MBq/body weight kg at the centers that used a C-PET scanner.

†Administred activity was reported as the amount per body weight MBq/kg; eg, 360 MBq was administered to a 60 kg patient for 6 MBq/kg).

‡Mean ± standard deviation.

three retrospective studies.[14,36,37] Physicians' knowledge of interim PET results may affect their assessment of patients' response as well as their treatment decisions, introducing biases.[39] Only three prospective studies[13,29,35] explicitly adopted blinding of clinicians to interim PET results to deal with these biases. In three prospective studies,[14,30,31] although they did not explicitly report the use of blinding, interim PET was not utilized to alter the preplanned treatment strategies. In two retrospective studies,[32,38] interim PET results had no effect on the treatment decisions. Because the assessment of treatment failure is not always objective, the absence of blinding can still potentially influence the way treating physicians judge the final clinical outcome in favor of interim PET, especially when the outcome is equivocal.[11,39] Although all the studies adopted the standard guidelines on response assessment[40,41] as the reference standard, they did not specify minimum follow-up period or situations where pathological confirmation was required. Four studies[29,32,33,38] employed post-therapy or follow-up PET to complement post-therapy response assessment. Because post-therapy response assessment with PET is still imperfect,[9] the applied reference standard could overestimate prognostic accuracy.[39]

Sensitivity, Specificity, LRs, and Summary ROC Curves

For advanced-stage HL, studies reported sensitivity from 0.67 to 1.00 and consistently high specificity from 0.94 to 1.00 for interim FDG-PET (Table 4; Fig 1). Summary estimates were 0.81 for sensitivity (95% CI, 0.72 to 0.89), 0.97 for specificity (95% CI, 0.94 to 0.99), 28.4 for positive LR (95% CI, 14.2 to 56.7), and 0.19 for negative LR (95% CI, 0.12 to 0.30). We did not estimate summary ROC curves because data points were closely clustered together with limited variations, a situation in which the hierarchical model could not produce reliable estimates (Fig 2).

DLBCL studies reported wide-ranging sensitivity (0.50 to 1.0) and specificity (0.73 to 1.00) values for interim FDG-PET (Table 4; Fig 1). Combined estimates had a sensitivity of 0.78 (95% CI, 0.64 to 0.87), a specificity of 0.87 (95% CI, 0.75 to 0.93), a positive LR of 5.9 (95% CI, 2.8 to 12.3), and a negative LR of 0.26 (95% CI, 0.15 to 0.46). The Q* statistic for the summary ROC curve was 0.82 (Fig 2).

In sensitivity analyses, the summary prognostic accuracy was stable for both advanced-stage HL and DLBCL regardless of how MRU results or early-censored cases without treatment failure were counted (results not shown). Regarding alternative reference standards based on the duration of clinical follow-up, subgroup data were available for five advanced-stage HL studies (n = 232)[13,14,30,32,37] and five DLBCL studies (n = 181)[12,32,35,36,38] (online-only Appendix Table A5). All DLBCL studies had improvement in sensitivity with loss of specificity when only progression during first-line therapy was counted by the alternative reference standard. A similar tendency was

Table 4. Study Results of Positron Emission Tomography for Interim Response Assessment of Malignant Lymphoma

Study	Year	Total No.	Progression or Relapse (%)	TP	FN	FP	TN	Sensitivity	95% CI	Specificity	95% CI	Positive Likelihood Ratio	95% CI	Negative Likelihood Ratio	95% CI
Advanced-stage HL															
Friedberg et al[33]	2004	22	23	4	1	1	16	0.80	0.28 to 1.00	0.94	0.71 to 1.00	13.6	1.9 to 95.7	0.21	0.04 to 1.23
Hutchings et al[37]	2005	28	32	6	3	0	19	0.67	0.30 to 0.93	1.00	0.82 to 1.00	26.0	1.6 to 416.8	0.36*	0.15 to 0.84
Gallamini et al[30]	2006	108	19	18	3	2	85	0.86	0.64 to 0.97	0.98	0.92 to 1.00	37.3	9.4 to 148.4	0.15	0.05 to 0.42
Hutchings et al[13]	2006	46	28	10	3	1	32	0.77	0.46 to 0.95	0.97	0.84 to 1.00	25.4	3.6 to 178.9	0.24	0.09 to 0.64
Kostakoglu et al[32]	2006	10	50	5	0	0	5	1.00	0.48 to 1.00	1.00	0.48 to 1.00	11.0	0.8 to 158.0	0.09	0.01 to 1.31
Zinzani et al[14]	2006	40	23	8	1	0	31	0.89	0.52 to 1.00	1.00	0.89 to 1.00	54.4	3.4 to 861.6	0.15†	0.04 to 0.67
Gallamini et al[29]	2007	106	20	15	4	4	83	0.79	0.54 to 0.94	0.95	0.89 to 0.99	17.2	6.4 to 46.0	0.22	0.09 to 0.53
DLBCL															
Spaepen et al[34]	2002	47	47	20	2	0	25	0.91	0.71 to 0.99	1.00	0.86 to 1.00	46.3	3.0 to 724.1	0.11	0.03 to 0.36
Haioun et al[31]	2005	83	39	20	12	14	37	0.63	0.44 to 0.79	0.73	0.58 to 0.84	2.3	1.4 to 3.8	0.52	0.32 to 0.83
Mikhaeel et al[12]	2005	57	38	15	7	8	27	0.68	0.45 to 0.86	0.77	0.60 to 0.90	3.0	1.5 to 5.8	0.41‡	0.22 to 0.78
Fruchart et al[35]	2006	35	29	9	1	6	19	0.90	0.56 to 1.00	0.76	0.55 to 0.91	3.8	1.8 to 7.8	0.13	0.02 to 0.86
Kostakoglu et al[32]	2006	24	38	9	0	1	14	1.00	0.66 to 1.00	0.93	0.68 to 1.00	10.1	2.2 to 46.8	0.06	0.00 to 0.83
Querellou et al[38]	2006	21	29	3	3	1	14	0.50	0.12 to 0.88	0.93	0.68 to 1.00	7.5	1.0 to 58.6	0.54	0.24 to 1.21
Ng et al[36]	2007	45	27	8	4	4	28	0.67	0.35 to 0.90	0.88	0.71 to 0.97	5.3	2.0 to 14.5	0.38	0.17 to 0.86

Abbreviations: DLBCL, diffuse large B-cell lymphoma; FN, false negative; FP, false positive; MRU, minimal residual uptake; TN, true negative; TP, true positive.
*The likelihood ratios for a MRU and a negative scan were 0.35 (95% CI, 0.05 to 2.5) and 0.33 (95% CI, 0.09 to 1.1), respectively, if these two categories were estimated separately.
†The likelihood ratios for a MRU and a negative scan were 1.1 (95% CI, 0.14 to 9.7) and 0.06 (95% CI, 0.00 to 0.84), respectively, if these two categories were estimated separately.
‡The likelihood ratios for a MRU and a negative scan were 0.96 (95% CI, 0.25 to 3.6) and 0.29 (95% CI, 0.12 to 0.73), respectively, if these two categories were estimated separately.

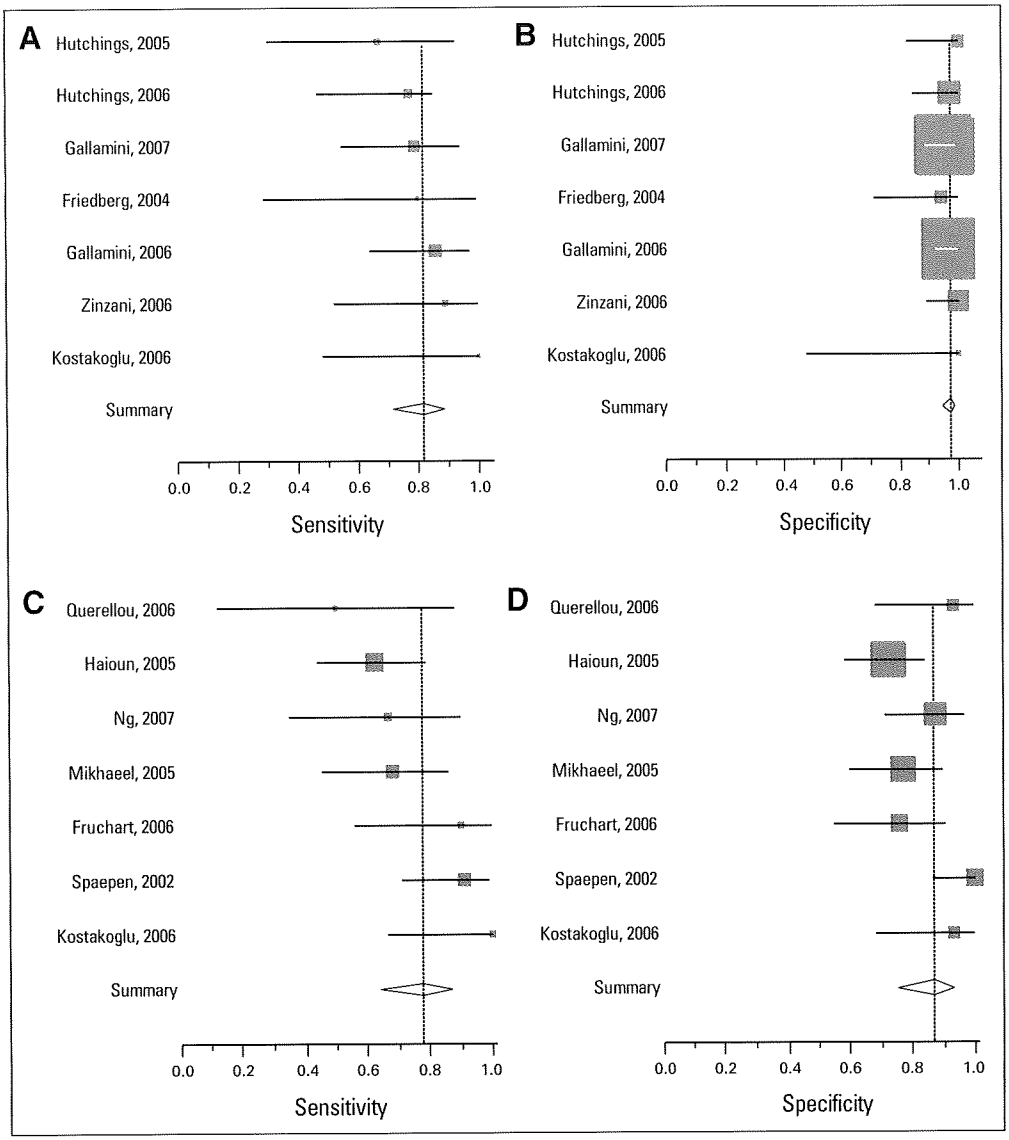

Fig 1. Sensitivity and specificity for (A, B) advanced-stage Hodgkin's lymphoma and (C, D) diffuse large B-cell lymphoma. The size of the square plotting is proportional to the number of patients with treatment failure for sensitivity and in remission for specificity. The horizontal lines are the 95% CIs. The vertical lines represent the summary estimates.

observed in all but one[30] advanced-stage HL studies (online-only Appendix Fig A2).

Subgroup Analyses and Meta-Regression Analyses

We did not perform subgroup analyses for advanced-stage HL because there were too few data points and there was little variation of the results across studies (Fig 1). Visual assessment of the ROC plots of DLBCL studies did not identify meaningful subgroups (data not shown). Meta-regression analyses on both advanced-stage HL and DLBCL did not find any clinical or test characteristics to explain the observed variability (data not shown).

DISCUSSION

This systematic review of interim response assessment of FDG-PET for patients with untreated advanced-stage HL showed that studies consistently reported high specificity and positive LRs. Although study quality was limited in some studies, as demographic and clinical characteristics of included patients were reasonably comparable over the studies, our results should generally be applicable to adult and adolescent patients with low- to intermediate-risk (IPS 0 to 3) receiving standard full course ABVD or comparable regimens. Because the summary positive LR is very high, positive PET results after a few cycles of chemotherapy would probably have an excellent ability to predict poor responders. Patients with negative PET, which predicts good response during the therapy, still have a moderate risk of post-treatment relapse since the summary negative LR is 0.19.[42]

The reported sensitivity and specificity of DLBCL studies of interim FDG-PET varied. This review also identified considerable clinical heterogeneity in these studies. For example, studies included patients with varied risk of treatment failure and adopted various therapeutic interventions. Also, studies were heterogeneous in how PET was used, such as the number of chemotherapy cycles before PET

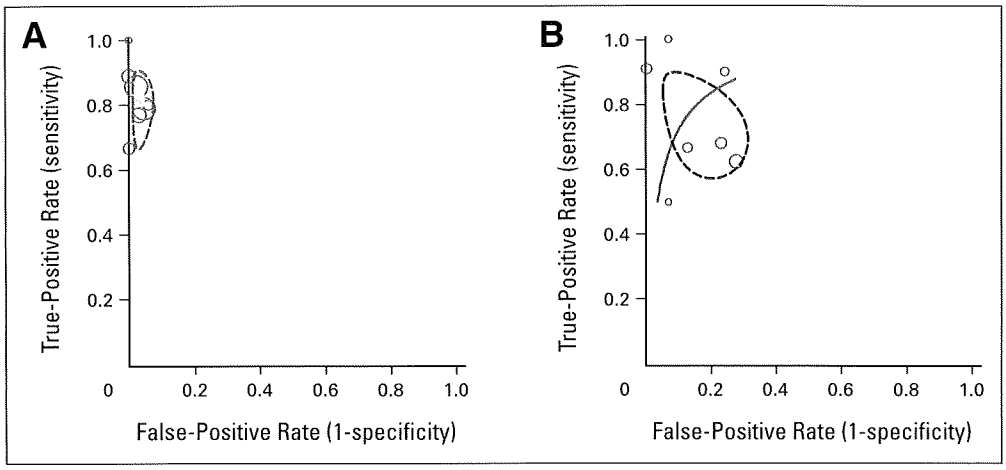

Fig 2. Receiver operating characteristic (ROC) plotting for (A) advanced-stage Hodgkin's lymphoma and (B) diffuse large B-cell lymphoma. Individual study estimates of sensitivity and 1 − specificity are shown (open circles). Summary ROC curve is presented only for DLBCL. Closed square represents the summary estimates. Dashed boundary represents the 95% confidence region for the summary sensitivity and specificity.

and the timing of scanning during the chemotherapy cycle.[43] Thus, our summary estimates should be interpreted carefully. Although we performed subgroup analyses and meta-regression analyses, we could not identify characteristics to explain the variability.

This study has several important limitations. Because only 13 studies with pertinent data were included in the meta-analysis, it may lack the power to detect clinically meaningful factors. In sensitivity analyses, fewer studies were available; therefore, the results may be less reliable. Although we did not independently estimate the summary LR for a MRU result, this distinct category may carry a worse prognosis than a clearly negative scan as reported.[12,14] Also, our results are likely subject to overestimation due to methodologic limitations in original studies, such as the absence of blinding of interim PET results to clinicians to assess final clinical outcomes.[11] Further, because of lack of data, we did not address the comparison between FDG-PET and CT or FDG-PET/CT and PET alone[38]; this review cannot answer whether PET is better than CT or whether the combined modality is superior to stand alone PET. In addition, this review did not specifically focus on limited-stage lymphoma; thus our results cannot answer the clinical question of whether early-interim PET can reliably identify good responders with localized disease. Finally, although three advanced-stage HL studies[13,29,30] and one DLBCL study[31] reported interim FDG-PET scan as a statistically significant independent prognostic factor in addition to IPS and IPI, respectively, we did not directly address this issue. For advanced-stage HL, because the included studies had few poor-risk (IPS 4 to 7) patients, our results may be less applicable to high-risk populations.

Interim PET should remain at this time as a test to be evaluated as part of clinical research where treatment regimens and imaging conditions are standardized; thus it should not be employed in the routine setting. This review supports conducting prospective trials for advanced-stage HL patients especially with low- to intermediate-risk (IPS 0 to 3) that incorporate early altering treatment to more intensive approach on the basis of positive FDG-PET results. For DLBCL, there is insufficient data to support similar trials. Additional prospective prognostic accuracy studies in the setting of conventional strategy would be needed to elucidate subgroups and timings of interim PET to better identify poor responders. Also, outside of study protocols where treatment strategies are explicitly defined on the basis of scan results, biopsy should be considered for positive PET findings if they are used to prompt a change in patient management. This is especially relevant if there is discrepancy between the scan results and other clinical data. Although biopsy cannot provide quantitative information as to how much residual tumor exists, it still is the most reliable way to confirm the presence of disease.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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REFERENCES

1. Jemal A, Siegel R, Ward E, et al: Cancer statistics, 2007. CA Cancer J Clin 57:43-66, 2007
2. Connors JM: State-of-the-art therapeutics: Hodgkin's lymphoma. J Clin Oncol 23:6400-6408, 2005
3. Coiffier B: State-of-the-art therapeutics: Diffuse large B-cell lymphoma. J Clin Oncol 23:6387-6393, 2005
4. Hasenclever D, Diehl V: A prognostic score for advanced Hodgkin's disease: International Prognostic Factors Project on advanced Hodgkin's disease. N Engl J Med 339:1506-1514, 1998
5. The International Non-Hodgkin's Lymphoma Prognostic Factors Project: A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med 329:987-994, 1993
6. Diehl V, Franklin J, Pfreundschuh M, et al: Standard and increased-dose BEACOPP chemotherapy compared with COPP-ABVD for advanced Hodgkin's disease. N Engl J Med 348:2386-2395, 2003
7. Greb A, Bohlius J, Trelle S, et al: High-dose chemotherapy with autologous stem cell support in first-line treatment of aggressive non-Hodgkin lymphoma: Results of a comprehensive meta-analysis. Cancer Treat Rev 33:338-346, 2007
8. Seam P, Juweid ME, Cheson BD: The role of FDG-PET scans in patients with lymphoma. Blood 110:3507-3516, 2007
9. Terasawa T, Nihashi T, Hotta T, et al: Fluorine-18-Fluorodeoxyglucose positron emission tomography for post-therapy assessment of Hodgkin's lymphoma and aggressive non-Hodgkin's lymphoma: A systematic review. J Nucl Med 49:13-21, 2008
10. Schelbert HR, Hoh CK, Royal HD, et al: Society of Nuclear Medicine Procedure Guideline for Tumor Imaging Using F-18 FDG version 2.0. 1999. http://interactive.snm.org/index.cfm?PageID=772&RPID=969
11. Whiting P, Rutjes AW, Reitsma JB, et al: The development of QUADAS: A tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews. BMC Med Res Methodol 3:25, 2003
12. Mikhaeel NG, Hutchings M, Fields PA, et al: FDG-PET after two to three cycles of chemotherapy predicts progression-free and overall survival in high-grade non-Hodgkin lymphoma. Ann Oncol 16:1514-1523, 2005
13. Hutchings M, Loft A, Hansen M, et al: FDG-PET after two cycles of chemotherapy predicts treatment failure and progression-free survival in Hodgkin lymphoma. Blood 107:52-59, 2006
14. Zinzani PL, Tani M, Fanti S, et al: Early positron emission tomography (PET) restaging: A predictive final response in Hodgkin's disease patients. Ann Oncol 17:1296-1300, 2006
15. Gatsonis C, Paliwal P: Meta-analysis of diagnostic and screening test accuracy evaluations: Methodologic primer. Am J Roentgenol 187:271-281, 2006
16. Rabe-Hesketh S, Skrondal A, Pickles A: GLLAMM Manual. U.C. Berkeley Division of Biostatistics Working Paper Series. Working Paper 160, 2004. http://www.bepress.com/ucbbiostat/paper160/
17. Harbord RM, Deeks JJ, Egger M, et al: A unification of models for meta-analysis of diagnostic accuracy studies. Biostatistics 8:239-251, 2007
18. Kostakoglu L, Coleman M, Leonard JP, et al: PET predicts prognosis after 1 cycle of chemotherapy in aggressive lymphoma and Hodgkin's disease. J Nucl Med 43:1018-1027, 2002
19. Itti E, Haioun C, Rahmouni A, et al: Aggressive large cell lymphoma: Early assessment of therapeutic efficacy by positron emission tomography. Medecine Nucleaire 28:327-332, 2004
20. Dupuis J, Gaulard P, Hemery F, et al: Respective prognostic values of germinal center phenotype and early (18)fluorodeoxyglucose-positron emission tomography scanning in previously untreated patients with diffuse large B-cell lymphoma. Haematologica 92:778-783, 2007
21. Romer W, Hanauske AR, Ziegler S, et al: Positron emission tomography in non-Hodgkin's lymphoma: Assessment of chemotherapy with fluorodeoxyglucose. Blood 91:4464-4471, 1998
22. Slaby J, Belohlavek O, Taborska K, et al: Predictive features of positron emission tomography after two cycles of induction therapy in malignant lymphoma. Cas Lek Cesk 141:312-315, 2002
23. Zijlstra JM, Hoekstra OS, Raijmakers PG, et al: 18FDG positron emission tomography versus 67Ga scintigraphy as prognostic test during chemotherapy for non-Hodgkin's lymphoma. Br J Haematol 123:454-462, 2003
24. Hoekstra OS, van Lingen A, Ossenkoppele GJ, et al: Early response monitoring in malignant lymphoma using fluorine-18 fluorodeoxyglucose single-photon emission tomography. Eur J Nucl Med 20:1214-1217, 1993
25. Hoekstra OS, Ossenkoppele GJ, Golding R, et al: Early treatment response in malignant lymphoma, as determined by planar fluorine-18-fluorodeoxyglucose scintigraphy. J Nucl Med 34:1706-1710, 1993
26. Torizuka T, Nakamura F, Kanno T, et al: Early therapy monitoring with FDG-PET in aggressive non-Hodgkin's lymphoma and Hodgkin's lymphoma. Eur J Nucl Med Mol Imaging 31:22-28, 2004
27. Jerusalem G, Beguin Y, Fassotte MF, et al: Persistent tumor 18F-FDG uptake after a few cycles of polychemotherapy is predictive of treatment failure in non-Hodgkin's lymphoma. Haematologica 85:613-618, 2000
28. Schot B, van Imhoff G, Pruim J, et al: Predictive value of early 18F-fluoro-deoxyglucose positron emission tomography in chemosensitive relapsed lymphoma. Br J Haematol 123:282-287, 2003
29. Gallamini A, Hutchings M, Rigacci L, et al: Early interim 2-[18F]fluoro-2-deoxy-D-glucose positron emission tomography is prognostically superior to international prognostic score in advanced-stage Hodgkin's lymphoma: A report from a joint Italian-Danish study. J Clin Oncol 25:3746-3752, 2007
30. Gallamini A, Rigacci L, Merli F, et al: The predictive value of positron emission tomography scanning performed after two courses of standard therapy on treatment outcome in advanced stage Hodgkin's disease. Haematologica 91:475-481, 2006
31. Haioun C, Itti E, Rahmouni A, et al: [18F]fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) in aggressive lymphoma: An early prognostic tool for predicting patient outcome. Blood 106:1376-1381, 2005
32. Kostakoglu L, Goldsmith SJ, Leonard JP, et al: FDG-PET after 1 cycle of therapy predicts outcome in diffuse large cell lymphoma and classic Hodgkin disease. Cancer 107:2678-2687, 2006
33. Friedberg JW, Fischman A, Neuberg D, et al: FDG-PET is superior to gallium scintigraphy in staging and more sensitive in the follow-up of patients with de novo Hodgkin lymphoma: A blinded comparison. Leuk Lymphoma 45:85-92, 2004
34. Spaepen K, Stroobants S, Dupont P, et al: Early restaging positron emission tomography with (18)F-fluorodeoxyglucose predicts outcome in patients with aggressive non-Hodgkin's lymphoma. Ann Oncol 13:1356-1363, 2002
35. Fruchart C, Reman O, Le Stang N, et al: Prognostic value of early 18 fluorodeoxyglucose positron emission tomography and gallium-67 scintigraphy in aggressive lymphoma: A prospective comparative study. Leuk Lymphoma 47:2547-2557, 2006
36. Ng AP, Wirth A, Seymour JF, et al: Early therapeutic response assessment by (18)FDG-positron emission tomography during chemotherapy in patients with diffuse large B-cell lymphoma: Isolated residual positivity involving bone is not usually a predictor of subsequent treatment failure. Leuk Lymphoma 48:596-600, 2007
37. Hutchings M, Mikhaeel NG, Fields PA, et al: Prognostic value of interim FDG-PET after two or three cycles of chemotherapy in Hodgkin lymphoma. Ann Oncol 16:1160-1168, 2005
38. Querellou S, Valette F, Bodet-Milin C, et al: FDG-PET/CT predicts outcome in patients with aggressive non-Hodgkin's lymphoma and Hodgkin's disease. Ann Hematol 85:759-767, 2006
39. Whiting P, Rutjes AW, Reitsma JB, et al: Sources of variation and bias in studies of diagnostic accuracy: A systematic review. Ann Intern Med 140:189-202, 2004
40. Cheson BD, Horning SJ, Coiffier B, et al: Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas: NCI Sponsored International Working Group. J Clin Oncol 17:1244-1253, 1999
41. Lister TA, Crowther D, Sutcliffe SB, et al: Report of a committee convened to discuss the evaluation and staging of patients with Hodgkin's disease: Cotswolds meeting. J Clin Oncol 7:1630-1636, 1989
42. Grimes DA, Schulz KF: Refining clinical diagnosis with likelihood ratios. Lancet 365:1500-1505, 2005
43. Juweid ME, Stroobants S, Hoekstra OS, et al: Use of positron emission tomography for response assessment of lymphoma: Consensus of the Imaging Subcommittee of International Harmonization Project in Lymphoma. J Clin Oncol 25:571-578, 2007

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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.

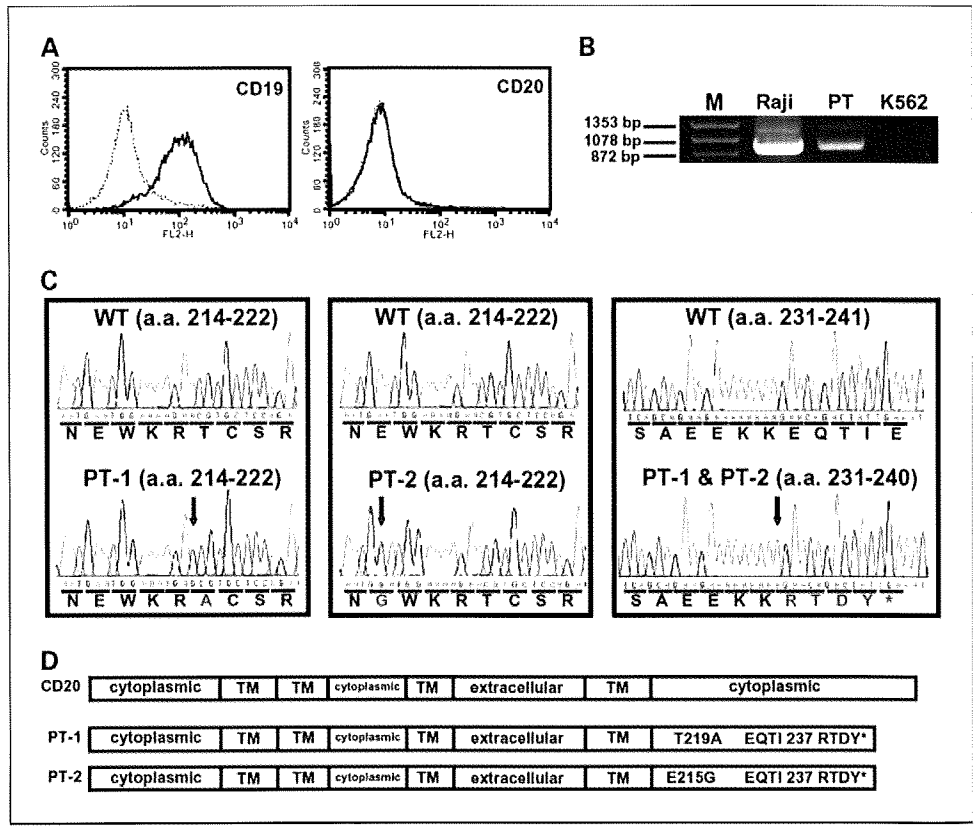

Fig. 1. CD20 expression and mutation analysis in CD19-positive lymphoma cells from a patient with posterior mediastinal lymphoma. *A,* flow cytometry for CD19 and CD20 cells. *B,* RNA analysis. RT-PCR using CD20 primers. Total RNAs from Raji and K562 cells were used as positive and negative controls, 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.

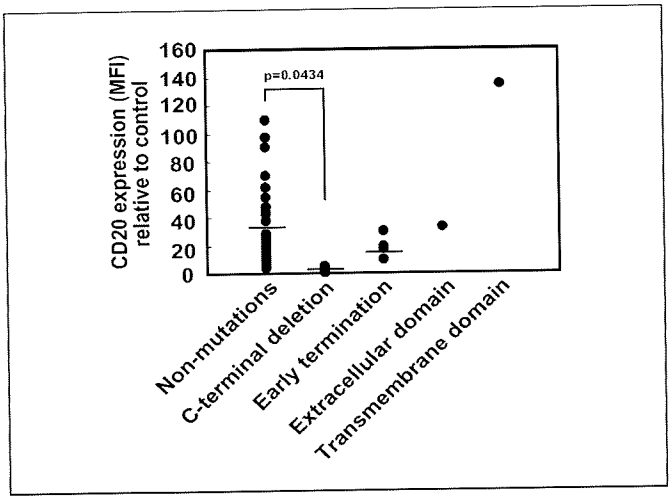

Fig. 2. Relationship between mutations and CD20 expression. Mean fluorescence intensity of CD20 relative to the control was assessed in CD19-positive cells from clinical samples by flow cytometric analysis. Fifty cases were classified as nonmutations or as mutations and grouped according to the domain 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.

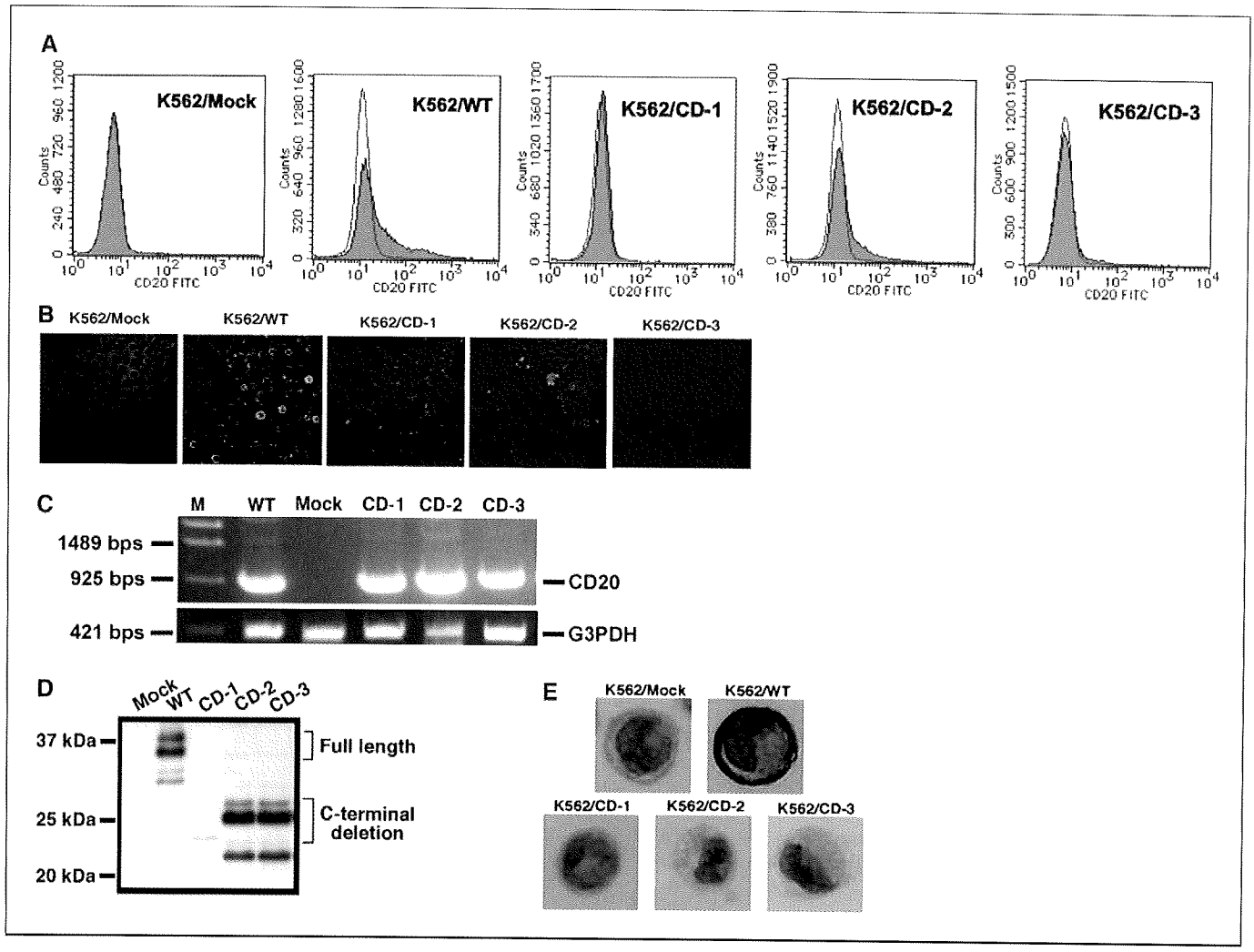

Fig. 3. CD20 expression in mutants CD-1, CD-2, and CD-3. Wild-type and mutant sequences were stably transfected into K562 cells (K562/mock, K562/WT, K562/CD-1, K562/CD-2, and K562/CD-3); after which, flow cytometric analysis was done with Alexa Fluor 488–labeled rituximab, and confocal laser scanning 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.

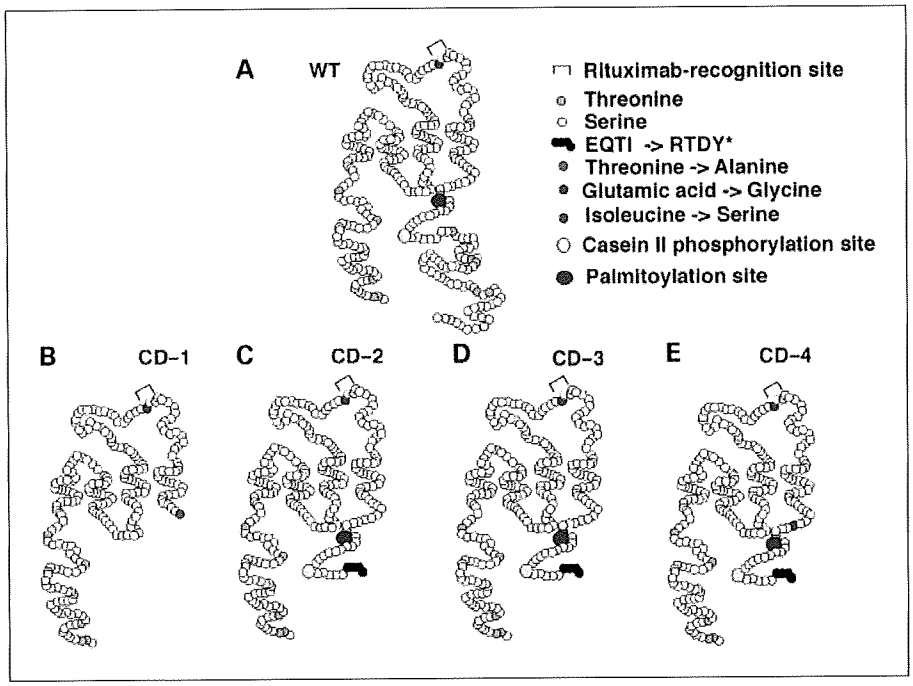

Fig. 4. The structures of wild-type and mutant CD20. Wild-type CD20 is composed of 297 amino acids (*A*), and casein kinase 2 and calcium/calmodulin-dependent protein kinase 2 phosphorylation sites are expected in its C-terminal cytoplasmic domain. C-terminal deletion mutants are shown as CD-1 (*B*),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

1. Arin MJ, Hunzelmann N. Anti-B-cell-directed immunotherapy (rituximab) in the treatment of refractory pemphigus—an update. Eur J Dermatol 2005;15: 224–30.
2. Emens LA. Trastuzumab: targeted therapy for the management of HER-2/neu-overexpressing metastatic breast cancer. Am J Ther 2005;12:243–53.
3. Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun 2005;333:328–35.
4. Vasir JK, Labhasetwar V. Targeted drug delivery in cancer therapy. Technol Cancer Res Treat 2005;4: 363–74.
5. Coiffier B. State-of-the-art therapeutics: diffuse large B-cell lymphoma. J Clin Oncol 2005;23:6387–93.
6. Schmits R, Schmitz N, Pfreundschuh M. The best treatment for diffuse large B-cell lymphoma: a German perspective. Oncology (Huntingt) 2005;19:16–25.
7. Hagenbeek A, Lewington V. Report of a European consensus workshop to develop recommendations for the optimal use of (90)Y-ibritumomab tiuxetan (Zevalin) in lymphoma. Ann Oncol 2005;16:786–92.
8. Haidar JH, Shamseddine A, Salem Z, et al. Loss of CD20 expression in relapsed lymphomas after rituximab therapy. Eur J Haematol 2003;70:330–2.
9. Alvaro-Naranjo T, Jaen-Martinez J, Guma-Padro J, Bosch-Princep R, Salvado-Usach MT. CD20-negative DLBCL transformation after rituximab treatment in follicular lymphoma: a new case report and review of the literature. Ann Hematol 2003;82:585–8.
10. Rawal YB, Nuovo GJ, Frambach GE, Porcu P, Baiocchi RA, Magro CM. The absence of CD20 messenger RNA in recurrent cutaneous B-cell lymphoma following rituximab therapy. J Cutan Pathol 2005;32: 616–21.
11. Smith MR. Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance. Oncogene 2003;22:7359–68.
12. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
13. Metaxa-Mariatou V, Papadopoulos S, Papadopoulou E, et al. Molecular analysis of GISTs: evaluation of sequencing and dHPLC. DNA Cell Biol 2004;23: 777–82.
14. Willmore C, Holden JA, Zhou L, Tripp S, Wittwer CT, Layfield LJ. Detection of c-kit-activating mutations in gastrointestinal stromal tumors by high-resolution amplicon melting analysis. Am J Clin Pathol 2004; 122:206–16.
15. Bellosillo B, Villamor N, Lopez-Guillermo A, et al. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated *in vitro* by a caspase-independent mechanism involving the generation of reactive oxygen species. Blood 2001;98:2771–7.
16. Takei K, Yamazaki T, Sawada U, Ishizuka H, Aizawa S. Analysis of changes in CD20, CD55, and CD59 expression on established rituximab-resistant B-lymphoma cell lines. Leuk Res 2006;30:635–1.
17. Terui Y, Sakurai T, Mishima Y, et al. Blockade of bulky lymphoma-associated CD55 expression by RNA interference overcomes resistance to complement-dependent cytotoxicity with rituximab. Cancer Sci 2006;97:72–9.
18. Jazirehi AR, Vega MI, Bonavida B. Development of rituximab-resistant lymphoma clones with altered cell signaling and cross-resistance to chemotherapy. Cancer Res 2007;67:1270–81.
19. Cragg MS, Walshe CA, Ivanov AO, Glennie MJ. The biology of CD20 and its potential as a target for mAb therapy. Curr Dir Autoimmun 2005;8:140–74.
20. Riley JK, Sliwkowski MX. *CD20*: a gene in search of a function. Semin Oncol 2000;27:17–24.
21. Polyak MJ, Tailor SH, Deans JP. Identification of a cytoplasmic region of CD20 required for its redistribution to a detergent-insoluble membrane compartment. J Immunol 1998;161:3242–8.
22. Hunter GK, Goldberg HA. Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. Biochem J 1994;302: 175–9.
23. Shankar G, GadekTR, Burdick DJ, Davison I, Mason WT, Horton MA. Structural determinants of calcium signaling by RGD peptides in rat osteoclasts: integrin-dependent and -independent actions. Exp Cell Res 1995;219:364–71.
24. DeBenedette M, Snow EC. Induction and regulation of casein kinase II during B lymphocyte activation. J Immunol 1991;147:2839–45.
25. Odegard VH, Schatz DG. Targeting of somatic hypermutation. Nat Rev Immunol 2006;6:573–83.
26. Lalonde R, Strazielle C. Spontaneous and induced mouse mutations with cerebellar dysfunctions: behavior and neurochemistry. Brain Res 2007;1140: 51–74.
27. Ragg S, Xu-Welliver M, Bailey J, et al. Direct reversal of DNA damage by mutant methyltransferase protein protects mice against dose-intensified chemotherapy and leads to *in vivo* selection of hematopoietic stem cells. Cancer Res 2000;60:5187–95.
28. Inoue K, Kohno T, Takakura S, Hayashi Y, Mizoguchi H, Yokota J. Frequent microsatellite instability and BAX mutations in T cell acute lymphoblastic leukemia cell lines. Leuk Res 2000;24:255–62.
29. Tomita A, Hiraga J, Kiyoi H, et al. Epigenetic regulation of CD20 protein expression in a novel B-cell lymphoma cell line, RRBL1, established from a patient treated repeatedly with rituximab-containing chemotherapy. Int J Hematol 2007;86:49–57.
30. Czuczman MS, Olejniczak S, Gowda A, et al. Acquirement of rituximab resistance in lymphoma cell lines is associated with both global *CD20* gene and protein down-regulation regulated at the pretranscriptional and posttranscriptional levels. Clin Cancer Res 2008;14:1561–70.
31. Olejniczak SH, Hernandez-Ilizaliturri FJ, Clements JL, Czuczman MS. Acquired resistance to rituximab is associated with chemotherapy resistance resulting from decreased Bax and Bak expression. Clin Cancer Res 2008;14:1550–60.
32. Solal-Celigny P, Roy P, Colombat P, et al. Follicular lymphoma international prognostic index. Blood 2004;104:1258–65.
33. Sweetenham JW. Diffuse large B-cell lymphoma: risk stratification and management of relapsed disease. Hematology Am Soc Hematol Educ Program. 2005:252–9.
34. Lossos IS, Morgensztern D. Non-Hodgkin's lymphoma in the microarray era. Clin Lymphoma 2004;5: 128–9.
35. Rossi D, Gaidano G. Molecular heterogeneity of diffuse large B-cell lymphoma: implications for disease management and prognosis. Hematology 2002;7: 239–52.
36. Ruiz-Vela A, Piqueras R, Carvalho-Pinto C, et al. ZAP-70 upregulation in transformed B cells after early pre-BI cell transplant into NOD/SCID mice. Oncogene 2005;24:5119–24.

Monitoring serum hepatitis C virus (HCV) RNA in patients with HCV-infected CD20-positive B-cell lymphoma undergoing rituximab combination chemotherapy

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Several studies have shown that the frequency of hepatitis C virus (HCV) infection is high in patients with B-cell non-Hodgkin's lymphoma (NHL). In these studies, liver dysfunction during chemotherapy has been demonstrated, but changes in HCV ribonucleic acid (RNA) levels during chemotherapy have not been well documented. In this study, we monitored serum HCV RNA levels and liver function in five HCV-infected patients with B-cell NHL undergoing treatment with rituximab-combination chemotherapy. Increased HCV RNA levels during or after the chemotherapy were observed in all five patients, and a significant increase in transaminases was seen in one case. In this case, serum HCV RNA level dramatically decreased at the time of the increase of transaminases, and this suggested that the cause of liver damage was an immune reaction against hepatocytes with HCV and not any anticancer drug induced liver toxicity. Monitoring of serum HCV RNA levels and transaminases may be helpful to understand the cause of liver dysfunction in patients receiving chemotherapy. However, increases of HCV viral load were not associated with the occurrence of liver dysfunction in this study. Further studies will be necessary to investigate more fully the relationship between changes in HCV viral load and liver function during chemotherapy for HCV-infected patients. Am. J. Hematol. 83:59–62, 2008. © 2007 Wiley-Liss, Inc.

INTRODUCTION

Several studies have reported treatment for B-cell non-Hodgkin's lymphoma (NHL) in hepatitis C virus (HCV)-positive patients; there have also been reports of liver dysfunction related to chemotherapy and stem cell transplantation (SCT) [1-6]. However, changes in HCV load during chemotherapy have not been well studied, and the status of HCV during chemotherapy is unknown. On the other hand, reactivation in hepatitis B virus (HBV)-positive patients during or after chemotherapy is a well-known complication [7,8]. Some authors have recommended monitoring HBV deoxyribonucleic acid (DNA) level during treatment, because knowing the status of the virus would be helpful in assessing the risk of reactivation and making decisions about the use of anti-HBV drugs [9-11].

Recently rituximab, a chimeric mouse–human monoclonal antibody, has become widely used for treatment of CD20-positive B-cell NHL [12,13], and there have been reports of reactivation in HBV-positive patients [9,14,15]. However, reactivation of HCV virus during rituximab treatment has not been reported, and the influence of rituximab against HCV viral load is not well understood.

Here we monitored serum HCV ribonucleic acid (RNA) levels and transaminases in HCV-positive patients receiving rituximab combination chemotherapy. We analyzed the relationship between changes in HCV viral load and liver function during and after chemotherapy that included rituximab.

MATERIALS AND METHODS

Patients

Eighty-one patients with CD20-positive B-cell NHL receiving rituximab combination chemotherapy at our institute between April and October 2005 were enrolled in the study. Patients were eligible if they were older than 18 years, had not been treated with chemotherapy, and had a performance status of 0 to 1, according to the criteria of the Eastern Cancer Oncology Group (ECOG). Patients who had clinically relevant cardiac diseases and were positive for antibodies against human immunodeficiency virus (HIV)-1 and 2, were excluded from this study.

Virological studies and assessment of HCV serum markers

Prior to chemotherapy, all 81 patients enrolled were tested for HCV antibody with two commercially available third-generation immunoassay kits (Monolisa anti-HCV Plus, Sanofi Diagnostics Pasteur; and Axsym HCV, version 3.0, Abbott). The presence of HCV RNA was examined in serum samples from all patients with the HCV antibody. HCV RNA in serum was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) with Amplicor HCV v2.0 (Roche Diagnostics K.K., Tokyo, Japan). During and after chemotherapy, patients with HCV viremia were evaluated at monthly intervals for HCV load in the circulation by real-time RT-PCR using Amplicor HCV Monitor v2.0 (Roche Diagnostics K.K.). HCV genotype characterization was performed according to the previously described procedures [16,17]. HCV genotypes 1a, 1b, 2a, 2b, and 3a were determined by selective amplification with primers derived from the core gene sequences, according to a previously described method [17], with the modification that the original genotype 1b-specific antisense primer, no. 133, was replaced by no. 492 [16].

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TABLE I. Clinical, Pathological, and Virological Data in Five Patients Positive for HCV RNA

						Prior to treatment			Peak during treatment		
No.	Sex	Age	Histology	Treatment	HCV genotype	AST (U/l)	ALT (U/l)	HCV RNA (KIU/ml)	AST (U/l)	ALT (U/l)	HCV RNA (KIU/ml)
1	M	74	DLBCL	R-CHOP	2a	21	22	97	522	550	1,100
2	F	67	DLBCL	R-CHOP	1b	42	36	2,300	46	36	12,500
3	F	78	DLBCL	R-CEO	1b	45	36	440	40	36	3,500
4	M	62	MALT	R-CHO	1b	45	74	1,000	66	74	10,000
5	M	78	DLBCL	R-CHOP	1b	25	17	3,200	40	17	15,500

DLBCL, diffuse large B-cell lymphoma; MALT, extranodal-marginal zone lymphoma of the mucosa-associated lymphoid tissue; R-CHOP, rituximab, cyclophosphamide, vincristine and prednisolone; R-CEO, rituximab, cyclophosphamide, vincristine and epirubicin; R-CHO, rituximab, cyclophosphamide, doxorubicin and vincristine; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

The presence of HBsAg was determined by chemiluminescence immunoassay (Abbott Japan, Tokyo, Japan). The presence of antibodies to HIV-1/2 was determined by particle agglutination with a commercially available kit (Fijirebio, Tokyo, Japan).

Assessment of liver function

Tests for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and albumin in serum were performed prior to chemotherapy, at least once a week during rituximab treatment, and at 1–3 month intervals after rituximab treatment until the end of the follow-up period.

Histology and immunohistochemistry

All 81 cases in this study were analyzed by an experienced hematopathologist (T.K). Diagnosis of CD20-positive B-cell NHL was confirmed using hematoxylin and eosin and/or Giemsa-stained slides, and by immunohistochemistry with antibodies against CD3, CD5, CD10, CD20, Ki67, and Bcl-2. Cases were subclassified according to the World Health Organization (WHO) classification [18].

RESULTS

Patient characteristics

Eighty-one patients (56 males and 25 females) with a median age of 56 years (range 28–81 years) were enrolled. In 81 patients, the histology of 42 indicated diffuse large B-cell lymphoma (DLBCL); of 22, follicular lymphoma; of 14, an extranodal-marginal zone lymphoma of the mucosa-associated lymphoid tissue (MALT). The remaining three patients were shown to have mantle cell lymphoma.

Six (7.4%) out of the 81 patients were positive for HCV antibody, and five of the six patients with anti-HCV had detectable HCV RNA before chemotherapy. Although two patients had HBsAg, no patients were positive for both HCV antibody and HBsAg. In the five patients (3 males and 2 females) positive for HCV RNA, the median age was 74 years (range 62–78). The histology of four cases was indicative of DLBCL, and that in the remaining case (Case 4) indicated a MALT lymphoma. No patients with DLBCL had features of transformation from a low-grade B-cell lymphoma. In all five patients, lymphoma cells strongly expressed CD20, but were negative for CD3. Three patients (Cases 1, 4, and 5) had previously been transfused with blood products. One patient (Case 3) was known to have chronic hepatitis, but no patient had a previous clinical history of cirrhosis. No patient had received treatment for HCV infection. Liver biopsies were performed on one patient (Case 4) before treatment. This male patient had a Metavir score of A1F1 [19]. HCV genotypes were 2a in the patient (Case 1) who developed severe liver damage, and 1b in the other four patients (Table I).

Treatment and outcomes

All 81 patients received weekly rituximab plus a CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or CHOP-like regimen. In five patients with HCV infection, three (Cases 1, 2, and 5) received the CHOP regimen, Case 3 received a CEO regimen (cyclophosphamide, vincristine, and epirubicin), and Case 4 received a CHO regimen (cyclophosphamide, doxorubicin, and vincristine) (Table I). Rituximab was administered at a standard dose of 375 mg/m^2 once a week for 8 weeks. All patients received a total of eight cycles of rituximab infusion. CHOP chemotherapy was administered in six cycles in four cases, and one patient (Case 1) received just three cycles of CHOP due to liver dysfunction such as elevation of ALT and AST levels, and then radiotherapy was given at a total dose of 50 Gy. Median follow-up was 12 months, and all patients survived. Four patients achieved a complete response (CR), and the other (Case 4) had a partial response (PR).

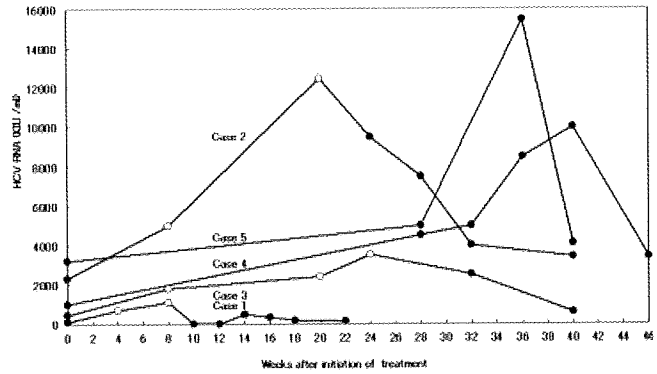

Figure 1. Changes of serum hepatitis C virus (HCV) RNA levels. The open circle represents serum HCV RNA level during chemotherapy, and the closed box denotes the serum HCV RNA level at baseline and after cessation of treatment.

HCV RNA and liver function

HCV RNA levels increased in all five patients during or after the start of chemotherapy (Fig. 1). Prior to chemotherapy, the serum HCV RNA level was 97–3,200 KIU/ml (Table I). In the four patients (Cases 2–5) who completed all six cycles of CHOP chemotherapy, the peak serum HCV RNA levels were 3,500–15,500 KIU/ml, and HCV RNA level decreased again at 5–9 months after the cessation of chemotherapy. In the remaining patient (Case 1) who did not complete chemotherapy, the peak HCV RNA level was 1,100 KIU/ml. In the four patients who completed treatment, Table I shows transaminase levels prior to chemotherapy and at peak levels during observation. AST and

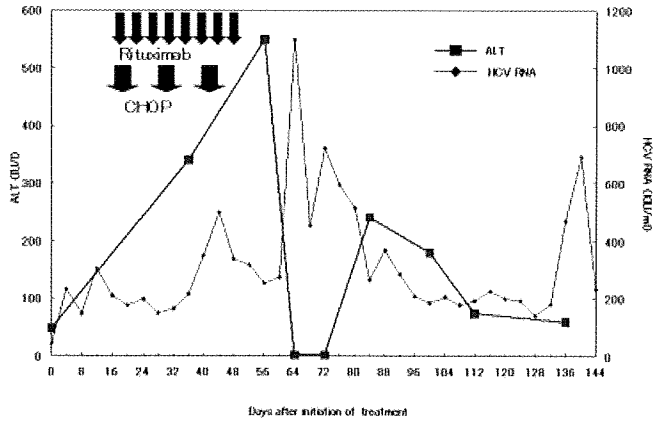

Figure 2. ALT level and HCV RNA titers in serum samples obtained periodically from Case 1. ALT, alanine aminotransferase; CHOP; cyclophosphamide, doxorubicin, vincristine and prednisone.

ALT levels at the start of chemotherapy were within a normal range (range AST, 10–30 IU/l; ALT, 5–35 IU/l) in two patients. Excluding Case 1, who had severe liver dysfunction, four patients exhibited a slight elevation of AST (40–70 IU/l) and ALT (10–80 IU/l). The severe liver dysfunction was observed in the patient with mild increase of HCV-RNA; the patients with a high increase of HCV replication did not experience liver dysfunction.

Clinical course of Case 1

Figure 2 shows the clinical course of Case 1, a 63-year-old man who developed DLBCL in October 2005. Prior to treatment, his liver function was normal and serum HCV RNA level was 97 KIU/ml. He received three cycles of CHOP chemotherapy and eight cycles of a weekly infusion of rituximab. After initial chemotherapy, serum AST and ALT levels increased gradually, and there was a parallel increase in HCV RNA level. Eight days after completion of the eighth infusion of rituximab, the HCV RNA level increased to a peak of 1,100 KIU/ml, but serum transaminase levels stayed at the level of less than 300 IU/l. One week later, however, the AST level abruptly increased to 550 IU/l and ALT to 552 IU/l, while the HCV RNA level decreased below the limit of detection, where it remained for the next week. The severe liver dysfunction in this patient led to the interruption of chemotherapy and hospitalization. While the patient recovered with supportive therapy, the ALT level remained elevated at 100 IU/l. The HCV RNA level increased again to a maximum of 320 KIU/ml, after its rapid decrease. A liver biopsy was not performed during this period. The patient received radiotherapy at a total dose of 33 Gy for treatment of the primary tumor. Two months later he achieved CR.

DISCUSSION

Numerous epidemiologic studies have described the high prevalence of HCV in patients with B-cell NHL. Besson et al. reported that the incidence of HCV infection was noted to be 0.5% of the total patients with DLBCL in Group d'Etude des Lymphomes de l'Adulte (GELA) programs [2]. The EPILYMPH study reported an incidence of 2.9% HCV infection in lymphoma patients, with a baseline population incidence of 2.3% [20]. However, these reports are mainly within geographic regions endemic for HCV. HCV viremia in patients undergoing chemotherapy has rarely been reported.

Liver dysfunction during or after chemotherapy is a rare complication in patients with HCV viremia compared with those with HBV viremia [2,5]. Some studies have found severe liver dysfunction in approximately 10% of HCV-positive patients with hematological malignancy. Another study revealed that 65% of the patients receiving chemotherapy for HCV-associated DLBCL had some hepatic toxicity [2]. They also demonstrated that in such patients, it was difficult to establish whether liver dysfunction was caused by drug toxicity or HCV reactivation [3-6]. In HCV-infected patients receiving SCT, hepatic complications are relatively common. Venoocculsive disease, graft-versus-host-disease (GVHD), viral infection, drug toxicity, or HCV reactivation are all mentioned as causes of liver dysfunction, though it is difficult to detect the actual cause of the particular liver dysfunction [21,22].

We monitored serum HCV RNA levels in five patients treated with rituximab combination chemotherapy, and all five showed a significant increase in HCV. Fong *et al.* [23] reported that short-term prednisone therapy induced HCV RNA elevation in 10 patients, and that this may have been caused by immunosuppression due to steroid hormones. However, two of our patients received regimens without prednisone, suggesting that increased HCV viremia was associated with not only steroid hormones, but also with anticancer agents or rituximab.

In the present study, we encountered a case with a marked increase of serum transaminase levels. The changes of serum HCV RNA and transaminase levels suggested that liver dysfunction was possibly due not to drug toxicity, but to the spread of HCV in the liver and to the immune reaction against HCV-infected hepatocytes. Similarly, a report of fulminant hepatitis in an HCV-positive patient receiving chemotherapy showed that HCV RNA dramatically decreased at the time of a hepatitis flare-up, and the authors concluded that the severe liver dysfunction was related to immune responses [24]. Monitoring of serum HCV RNA levels and transaminases may be helpful to understand the cause of liver dysfunction in patients receiving chemotherapy and SCT.

The addition of rituximab to chemotherapy did not seem to affect HBV-positive patients' tolerance to treatment [25]. There are reports of rituximab combination chemotherapy in HCV-positive patients; however, rituximab-related liver dysfunction has not been described [2,26]. Accelerated HCV replication has been noted in a case report of a patient with HCV-associated cirrhosis and lymphoma receiving rituximab; however, conclusions cannot be extrapolated to asymptomatic populations without significant additional data. Reports have indicated that without initial liver dysfunction, HCV-infected patients can experience an outcome similar to their HCV-counterparts when treated with standard chemoimmunotherapy in spite of differences in the presentation of the disease. All our patients received rituximab combined chemotherapy and liver dysfunction occurred in Case 1 after rituximab infusion, but we can not conclude that rituximab alone induced liver dysfunction since not all patients received rituximab monotherapy.

The HCV genotype was 2a only in the patient who developed severe liver damage, and was 1b in the remaining four patients. Recently, genotype 2c carriers in Italy have been shown to be at higher risk of virus reactivation than those with genotype 1b; moreover, genotype 2 is reported to be a risk factor for hepatitis virus reactivation in Taiwanese patients [27,28]. However, other genotypes have also been reported in patients with virus reactivation, and so the association between HCV reactivation and genotype 2a has not been clearly established [26]. On the other hand, HBV precore mutant, notably that with a unique G-to-A

mutation at position 1896 [29,30], has been established as a risk factor for fulminant hepatitis in patients receiving immunosuppressive agents [8]; administration of anti-HBV drugs are recommended in such cases. However, HCV has many genotype variants, and it is difficult to detect the specific mutant of reapplication. Further studies will be necessary to clarify this issue.

In conclusion, HCV RNA increased in all HCV-positive patients receiving rituximab combination chemotherapy. In one case with liver dysfunction, serum HCV RNA level dramatically decreased at the time of increase of transaminases, and immune reaction against HCV was suspected as a cause of liver dysfunction. Although the increase of HCV RNA level did not relate to a hepatitis flare-up, knowing the cause of liver dysfunction during chemotherapy will be very helpful for the management of that liver dysfunction. Unfortunately, liver biopsy was not done in our study, and this may allow for additional speculation with regard to etiology of rituximab-associated changes. More detailed investigations into HCV viral load and a larger sample size will be required to clarify the cause of liver dysfunction during immunosuppressive therapy.

References

1. Locasciulli A, Testa M, Pontisso P, et al. Hepatitis C virus genotypes and liver disease in patients undergoing allogeneic bone marrow transplantation. Bone Marrow Transplant 1997;19:237–240.
2. Besson C, Canioni D, Lepage E, et al. Characteristics and outcome of diffuse large B-cell lymphoma in hepatitis C virus-positive patients in LNH 93 and LNH 98 Groupe d'Etude des Lymphomes de l'Adulte programs. J Clin Oncol 2006;24:953–960.
3. Kanamori H, Fukawa H, Maruta A, et al. Case report: Fulminant hepatitis C viral infection after allogeneic bone marrow transplantation. Am J Med Sci 1992;303:109–111.
4. Zuckerman E, Zuckerman T, Douer D, et al. Liver dysfunction in patients infected with hepatitis C virus undergoing chemotherapy for hematologic malignancies. Cancer 1998;83:1224–1230.
5. Luppi M, Longo G, Ferrari MG, et al. Additional neoplasms and HCV infection in low-grade lymphoma of MALT type. Br J Haematol 1996;94:373–375.
6. Kawatani T, Suou T, Tajima F, et al. Incidence of hepatitis virus infection and severe liver dysfunction in patients receiving chemotherapy for hematologic malignancies. Eur J Haematol 2001;67:45–50.
7. Coiffier B. Hepatitis B virus reactivation in patients receiving chemotherapy for cancer treatment: Role of Lamivudine prophylaxis. Cancer Invest 2006;24:548–552.
8. Dai MS, Lu JJ, Chen YC, et al. Reactivation of precore mutant hepatitis B virus in chemotherapy-treated patients. Cancer 2001;92:2927–2932.
9. Hamaki T, Kami M, Kusumi E, et al. Prophylaxis of hepatitis B reactivation using lamivudine in a patient receiving rituximab. Am J Hematol 2001;68: 292–294.
10. Dai MS, Chao TY, Kao WY, et al. Delayed hepatitis B virus reactivation after cessation of preemptive lamivudine in lymphoma patients treated with rituximab plus CHOP. Ann Hematol 2004;83:769–774.
11. Tsutsumi Y, Kanamori H, Mori A, et al. Reactivation of hepatitis B virus with rituximab. Expert Opin Drug Saf 2005;4:599–608.
12. Coiffier B, Haioun C, Ketterer N, et al. Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: A multicenter phase II study. Blood 1998;92:1927–1932.
13. Vose JM, Link BK, Grossbard ML, et al. Phase II study of rituximab in combination with chop chemotherapy in patients with previously untreated, aggressive non-Hodgkin's lymphoma. J Clin Oncol 2001;19:389–397.
14. Sarrecchia C, Cappelli A, Aiello P. HBV reactivation with fatal fulminating hepatitis during rituximab treatment in a subject negative for HBsAg and positive for HBsAb and HBcAb. J Infect Chemother 2005;11:189–191.
15. Tsutsumi Y, Kanamori H, Minami H, et al. Successful treatment of lymphoma with fludarabine combined with rituximab after immune thrombocytopenia induced by fludarabine. Ann Hematol 2005;84:269–271.
16. Okamoto H, Kobata S, Tokita H, et al. A second-generation method of genotyping hepatitis C virus by the polymerase chain reaction with sense and antisense primers deduced from the core gene. J Virol Methods 1996;57:31–45.
17. Okamoto H, Tokita H, Sakamoto M, et al. Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. J Gen Virol 1993;74:2385–2390.
18. Jaffe E. S. HNL, Stein H, Vardiman JW, editors. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissue. 2001.
19. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 1996;24:289–293.
20. Nieters A, Kallinowski B, Brennan P, et al. Hepatitis C and risk of lymphoma: Results of the European multicenter case–control study EPILYMPH. Gastroenterology 2006;131:1879–1886.
21. Locasciulli A, Bruno B, Alessandrino EP, et al. Hepatitis reactivation and liver failure in haemopoietic stem cell transplants for hepatitis B virus (HBV)/hepatitis C virus (HCV) positive recipients: A retrospective study by the Italian group for blood and marrow transplantation. Bone Marrow Transplant 2003;31:295–300.
22. Maruta A, Kanamori H, Fukawa H, et al. Liver function tests of recipients with hepatitis C virus infection after bone marrow transplantation. Bone Marrow Transplant 1994;13:417–422.
23. Fong TL, Valinluck B, Govindarajan S, et al. Short-term prednisone therapy affects aminotransferase activity and hepatitis C virus RNA levels in chronic hepatitis C. Gastroenterology 1994;107:196–199.
24. Vento S, Cainelli F, Mirandola F, et al. Fulminant hepatitis on withdrawal of chemotherapy in carriers of hepatitis C virus. Lancet 1996;347:92–93.
25. Visco C, Arcaini L, Brusamolino E, et al. Distinctive natural history in hepatitis C virus positive diffuse large B-cell lymphoma: Analysis of 156 patients from northern Italy. Ann Oncol 2006;17:1434–1440.
26. Aksoy S, Abali H, Kilickap S, et al. Accelerated hepatitis C virus replication with rituximab treatment in a non-Hodgkin's lymphoma patient. Clin Lab Haematol 2006;28:211–214.
27. Rumi MG, De Filippi F, La Vecchia C, et al. Hepatitis C reactivation in patients with chronic infection with genotypes 1b and 2c: A retrospective cohort study of 206 untreated patients. Gut 2005;54:402–406.
28. Coppola N, Vatiero LM, Sagnelli E. HCV genotype 2 as a risk factor for reactivation of chronic HCV infection. Gut 2005;54:1207.
29. Carman WF, Jacyna MR, Hadziyannis S, et al. Mutation preventing formation of hepatitis B antigen in patients with chronic hepatitis B infection. Lancet 1989;2:588–591.
30. Okamoto H, Yotsumoto S, Akahane Y, et al. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J Virol 1990;64:1298–1303.

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ORIGINAL ARTICLE

Increased incidence of interstitial pneumonia by CHOP combined with rituximab

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Abstract Several authors have reported interstitial pneumonia (IP) during rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) therapy, while others have encountered *Pneumocystis jirovecii* pneumonia during rituximab-combined bi-weekly CHOP. Herein, we report that 13 of 90 (14%) patients developed IP during R-CHOP therapy, compared with none of 105 patients treated with CHOP alone as a historical control. There were no differences in baseline data between patients undergoing the two therapies. Among R-CHOP-treated patients, serum β-D-glucan was increased in 8 of 12 (75%) IP patients compared with none of 30 non-IP patients examined. In five IP patients who underwent sputum evaluation, two were positive for *P. jirovecii* by the polymerase chain reaction and another two were positive for *Candida albicans*. No other organisms were detected as causative pathogens. Treatment with steroids, sulfamethoxazole-trimethoprim (ST), and antifungals was effective. Our results suggest that R-CHOP raises the incidence of IP, possibly through increasing the susceptibility to *P. jirovecii* and fungal infection. The need for prophylactic antifungals and ST during R-CHOP should be evaluated by randomized controlled trials.

Keywords Interstitial pneumonia · Rituximab · R-CHOP · β-D-glucan

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1 Introduction

Rituximab, a chimeric mouse–human monoclonal antibody against CD20, is used for monotherapy, as well as combination chemotherapy, against CD20-positive B-cell non-Hodgkin's lymphoma (NHL), and its efficacy has been demonstrated with a low incidence of adverse effects [1]. There have been several case reports of interstitial pneumonia (IP) in patients treated with rituximab, and allergic mechanisms or increased susceptibilities to infection have been suggested [2–7]. Recently, IP has been shown to be caused by *Pneumocystis jirovecii* (*P. carinii* pneumonia, PCP) during treatment with rituximab plus bi-weekly cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP-14) with or without etoposide [8, 9]. These reports indicated that PCP occurred in the immunosuppressive state induced by rituximab or dose-intense agents. However, IP development in rituximab-treated patients has not been systematically analyzed, and its incidence and etiology in such patients therefore remain unclear.

Here, we compared the incidence of IP in 90 B-cell NHL patients treated with rituximab-combined CHOP (R-CHOP) with that in 105 similar patients treated with CHOP alone as a historical control. IP was diagnosed in 13 of 90 patients treated with R-CHOP compared with none of

the 105 patients receiving CHOP alone, and the incidence was significantly higher in R-CHOP-treated patients. The characteristics of IP patients were analyzed, and the possible etiology is discussed.

2 Patients and methods

A total of 90 patients with CD20-positive B-cell NHL undergoing R-CHOP therapy between April 2005 and October 2006 and 105 patients with B-cell NHL who received CHOP therapy between June 2000 and December 2004 at our institute were compared regarding their characteristics and the incidence of IP by *t*-tests and Fischer's exact test. The study protocol and sampling were approved by the Institutional Review Board of the Cancer Institute Hospital. Due to the retrospective nature of this study, written informed consent was not obtained from any patients.

Histological diagnosis was confirmed by an expert hematopathologist (K.T.), and staging was performed according to the Ann Arbor classification. For patients with stages Ib-IV, CHOP chemotherapy was repeated six times in both treatment groups, and a standard dose of rituximab (375 mg/m^2) was administered on day 1 of each cycle for patients who received R-CHOP. For patients with stage Ia, CHOP or R-CHOP chemotherapy was repeated for three cycles and rituximab was continued as for patients with stages Ib-IV, which was followed by radiotherapy. Antifungals and sulfamethoxazole-trimethoprim (ST) were not administered prophylactically to patients treated with either RCHOP or CHOP therapy, because PCP or fungal infection has not been proven to be aggravated by the addition of rituximab to CHOP.

We performed chest and abdominal computed tomography (CT) to evaluate the response of each lymphoma to treatment. Furthermore, chest X-rays and CT were carried out in patients who developed a high fever and dyspnea during or after treatment. IP was diagnosed on the basis of chest CT revealing bilateral diffuse pulmonary interstitial infiltrates, and hypoxia without hypercapnia on arterial blood gas analysis. In order to confirm the diagnosis, peripheral white blood cells (WBCs), serum lactate dehydrogenase (LDH), C-reactive protein (CRP), and Klebsvonden Lungen-6 (KL-6) were assessed in all patients who developed IP. We also monitored the serum immunoglobulin G (IgG) level in patients. As a test for infection, we measured serum β-D-glucan, antigens for *Candida*, *Aspergillus*, *Klebsiella*, and adenovirus, cytomegalovirus (CMV) antigenemia, and antibodies against *Mycoplasma*. Blood and sputum from some patients were cultured. We further analyzed the serum level of β-D-glucan in 30 patients receiving R-CHOP without any symptoms or signs suggesting IP during the same period as a control.

3 Results

Table 1 shows the characteristics of patients who received R-CHOP and CHOP, and the incidences of IP. There were no significant differences in the baseline characteristics, including the median age, sex, distribution of histology, disease stage, or International Prognostic Index between patients receiving the two treatments. IP was diagnosed in 13 of 90 (14.3%) patients treated with R-CHOP and none of 105 patients receiving CHOP. As other lung complications, two cases of bacterial pneumonia and one case of development of tuberculosis were detected in the R-CHOP group, and five cases of bacterial pneumonia in the CHOP group.

All IP cases during R-CHOP were diagnosed as outpatients. Furthermore, 12 of the cases occurred between 60 and 120 days after treatment initiation of (6, 1, 1, and 4 cases were diagnosed during the third, fourth, fifth, and sixth cycles of treatment, respectively). Chest radiographs showed bilateral diffuse ground-glass opacity in all patients. There was no cystic lesion, fibrotic area with honeycombing, or consolidation in air bronchograms. Patients developed severe hypoxia of 46.2–62.1 (mean 51.2) mmHg of PaO_2 without hypercapnia (mean $PaCO_2$; 35.4 mmHg, range 33.0–40.4), but no patients underwent a respiratory function test. No cases had a history of any other lung disease, disease progression nor lung involvement of the lymphoma at the onset of IP (Table 2).

At the onset of IP, the peripheral WBC counts were within the normal range in seven patients, elevated in two

Table 1 The characteristics of patients treated with both R-CHOP and CHOP

	R-CHOP $n = 90$ n (%)	CHOP $n = 105$ n (%)	P-value
Median age	66.5	68	0.2
Sex			
Male	46 (51)	60 (57)	0.4
Female	44 (49)	45 (43)	
Histology			
DLBCL	73 (81)	86 (82)	0.8
FL, others	17 (19)	19 (18)	
Stage			
I–II	48 (53)	56 (53)	0.9
III–IV	42 (47)	49 (47)	
IPI			
L, L-I	64 (71)	62 (59)	0.8
H, H-I	26 (29)	43 (41)	
IP	13 (14)	0	<0.05

R-CHOP rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone, *DLBCL* diffuse large B-cell lymphoma, *FL* follicular lymphoma, *IPI* International Prognosis Index, *L* low, *L-I* low-intermediate, *H* high, *H-I* high-intermediate, *IP* interstitial pneumonia