Cancer Science
The official journal of the Japanese Cancer Association

Prognostic impact of immunohistochemical biomarkers in diffuse large B-cell lymphoma in the rituximab era

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(Received April 15, 2009/Revised June 11, 2009/Accepted June 15, 2009/Online publication July 28, 2009)

We evaluated the usefulness of prognostic markers in patients with diffuse large B-cell lymphoma (DLBCL) treated with cyclophosphamide, vincristine, doxorubicin, and prednisolone (CHOP) ± rituximab (R-CHOP) in Japan. We studied 730 patients with DLBCL; 451 received CHOP and 279 R-CHOP. We analyzed biopsy samples immunohistochemically for markers of germinal center B cells (CD10, Bcl-6), postgerminal center B cells (Multiple myeloma-1), and apoptosis (Bcl-2). The median follow-up period for surviving patients was 56.4 months for the CHOP group and 25.2 months for the R-CHOP group. DLBCL were categorized as germinal center B (GCB) subtype (352/730; 48.2%) or non-GCB subtype (378/730; 51.8%). In the CHOP group, the high expression of CD10 ($P = 0.022$) or Bcl-6 ($P = 0.021$), or GCB subtype ($P = 0.05$) was associated with better overall survival, whereas the high expression of Bcl-2 ($P = 0.001$) or MUM1 ($P = 0.011$), or non-GCB subtype ($P = 0.05$) was associated with worse overall survival. In the R-CHOP group, however, these biomarkers except Bcl-6 were not significant prognostic factors. The patients with non-GCB subtype showed improved survival in the R-CHOP group ($P = 0.756$). The International Prognostic Index was a useful clinical marker of survival in the CHOP group ($P < 0.001$) and also in the R-CHOP group ($P < 0.001$). Results of improved survival with rituximab addition indicate that the relevance of previously recognized prognostic factors should be re-evaluated. (*Cancer Sci* 2009; 100: 1842–1847)

Diffuse large B-cell lymphoma (DLBCL) is one of the most common lymphoid neoplasms, characterized by heterogeneity in its clinical, immunophenotypic, and genetic features.[1,2] The cure rate with standard chemotherapy is as low as 30–40%.[3] Recently, the addition of the anti-CD20 monoclonal antibody, rituximab (R), to anthracycline-based chemotherapy such as the cyclophosphamide, vincristine, doxorubicin, and prednisolone (CHOP) regimen has been shown to improve the survival of DLBCL patients.[4–7] Despite these advances, responses to treatment are heterogeneous, and outcomes are often unpredictable. Furthermore, treatment is costly. These facts raise the need to identify more accurately those patients who would benefit from immunochemotherapy.

The International Prognostic Index (IPI) is considered to be the most important prognostic factor for survival and therefore the strongest indicator to identify high-risk patients who are unlikely to be cured with standard chemotherapy.[1] However, the five clinical characteristics that comprise the IPI score (i.e. age, Eastern Cooperative Oncology Group performance status, stage, extranodal involvement, and lactate dehydrogenase level) do not provide any information on the biological features of DLBCL cells, nor do they predict responsiveness to therapy. Therefore, there is a need for biomarkers that accurately predict outcome in these patients. Individual biomarkers may provide prognostic information for patients with DLBCL. For example, the expression levels of Bcl-2 and Bcl-6 have been associated with adverse and favorable outcomes in chemotherapy-treated patients, respectively.[2,8–13] Different studies, however, yield conflicting and inconclusive results, reflecting the heterogeneity of patient populations as well as technical factors related to staining, interpretation, and scoring of the data. In addition, single genes or molecules may simply be unable to reflect the heterogeneity of DLBCL accurately.

Recent studies have used gene expression profiling to identify the following three distinct subgroups of DLBCL: germinal center B-cell-like (GCB), activated B-cell-like, and primary mediastinal DLBCL.[2,14–16] Several prognostic models based on RNA or protein expression have been developed to predict survival in DLBCL patients.[17,18] However, a consensus regarding the stratification of DLBCL patients has not yet been achieved. Many studies have also focused on immunohistochemistry to identify risk groups because this technique avoids the limitations of using fresh tissue and is easy to carry out in routine clinical practice. Hans *et al.* proposed an algorithm based on the expression of CD10, Bcl-6, and Multiple myeloma-1 (MUM1)/

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doi: 10.1111/j.1349-7006.2009.01268.x

Table 1. Clinical characteristics in diffuse large B-cell lymphoma (DLBCL) patients treated with cyclophosphamide, vincristine, doxorubicin, and prednisolone (CHOP) and rituximab (R)-CHOP

Characteristic	Study population								
	Total			CHOP (n = 451)			R-CHOP (n = 279)		
	CHOP (%) (n = 451)	R-CHOP (%) (n = 279)	P-value	GCB (%) (n = 203)	Non-GCB (%) (n = 248)	P-value	GCB (%) (n = 149)	Non-GCB (%) (n = 130)	P-value
Male (%)	54.1	50.9	n.s.	58.1	50.8	n.s.	50.3	51.5	n.s.
Age (Mean)	64.4	65.4	n.s.	64.1	64.7	n.s.	65.1	65.8	n.s.
60 y or order (%)	65.0	69.2	n.s.	62.6	66.9	n.s.	67.8	70.8	n.s.
ECOG PS 2–4 (%)	20.6	23.3	n.s.	19.7	21.4	n.s.	22.8	23.9	n.s.
LDH > normal (%)	45.0	55.6	n.s.	49.3	59.7	<0.05	45.0	43.9	n.s.
Clinical Stage III/IV (%)	51.0	49.8	n.s.	45.3	55.7	<0.05	47.7	52.3	n.s.
No. extranordal sites > 1 (%)	17.1	20.1	n.s.	16.8	17.3	n.s.	25.3	22.3	n.s.
HI/high IPI (%)	39.5	36.2	n.s.	35.0	43.2	n.s.	32.9	40.0	n.s.

ECOG PS, Eastern Cooperative Oncology Group performance status; GCB, germinal center B-cell-like; high, high-risk group; HI, high–intermediate-risk group; IPI, International Prognostic Index; n.s., not significant.

Interferon regulatory factor-4 (IRF4) to discriminate between GCB and non-GCB DLBCL.[18]

In the present study, we therefore analyzed the data from 730 DLBCL patients treated with CHOP ± R in the Kyushu Lymphoma Study Group to determine whether these molecules remain valuable prognostic factors in the R era.

Materials and Methods

Patients. All of the patients (730 cases) were treated with curative intent with a CHOP-like (CHOP; n = 451) regimen or with CHOP + R (R-CHOP; n = 279) during the period 1995 to 2005, and clinical follow up was carried out until 31 December 2006 at the 22 hospitals of the Kyushu Lymphoma Study Group. All patients received anthracyclin-based regimens. The majority of the patients (94.0%) were treated with CHOP or cyclophosphamide, therarubicin, vincristine, and prednisolone (THP-COP), and few patients were treated with CHOP plus etoposide (CHOP-E) or methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisolone, and bleomycin (MACOP-B). Of the 730 patients, 68 received high-dose chemotherapy with autologous peripheral blood stem cell transplantation as a front-line therapy. The patients were enrolled based on the following criteria: diagnosis of *de novo* DLBCL, availability of paraffin-embedded tissue obtained at diagnosis before the initiation of therapy, and availability of follow-up and outcome data at the treating institutions. Primary mediastinal DLBCL and primary central nervous system lymphomas were not included in this study. Institutional review board approval was obtained from all of the participating institutions. Clinical staging of the disease according to the Ann Arbor classification[19] was determined by physical examination, bone marrow specimens, and computed tomography of the chest, abdomen, and pelvis. The following clinical and laboratory data were available at the time of diagnosis: age, sex, performance status, stage, number of extranodal sites involved, serum lactate dehydrogenase level, and the presence or absence of systemic ('B') symptoms. On the basis of the IPI scores, the patients were categorized into a low-, low–intermediate-, high–intermediate-, or high-risk group. None of the patients had a known history of human immunodeficiency virus infection or other forms of immunodeficiency. Follow-up information was obtained from patient medical records.

Histological sections were reviewed, and the diagnosis was confirmed as DLBCL according to the World Health Organization classification of hematopoietic tumors[20] by pathologists at each institute. Furthermore, all specimens were reviewed and reconfirmed at the Department of Pathology, Kurume University School of Medicine.

Immunohistochemistry. Staining and scoring for CD10 (clone 56C6; Novocastra, Newcastle, UK), Bcl-6 (clone P1F6; Novocastra), MUM1/IRF4 (clone MUM1p; DAKO, Glostrup, Denmark), and Bcl-2 (clone 124; DAKO) were carried out for all sections from each institute at the Department of Pathology, Kurume University School of Medicine, using standard immunohistochemistry methods.[21] The GCB and non-GCB DLBCL subtypes were classified as described by Hans *et al*.[18] The specimens were scored positive for CD10, Bcl-6, or MUM-1 staining if a minimum of 30% of the neoplastic cells was labeled,[18] whereas the cut-off level for Bcl-2 was 50%, in accordance with other reports.[10]

Statistical analysis. Survival curves were estimated using the product-limit method of Kaplan–Meier and were compared using the log-rank test. Multivariate analysis according to the Cox proportional hazards regression model,[22] with overall survival (OS) as the dependent variable, was used to adjust for the effects of immunohistochemical staining and IPI. The Mann–Whitney U-test or χ^2-test was used to compare the clinical outcome between various subgroups, and overall response (complete response + complete response unconfirmed) between treatment groups.[19] $P < 0.05$ was considered significant.

Results

Patient characteristics. To analyze the prognostic impact of immunohistochemical staining of the four markers for DLBCL, 451 DLBCL patients aged 23–88 years (median age, 66.0 years) were included in the CHOP group. The follow-up period ranged from 7 to 144 months (median, 44.4 months). For the R-CHOP group, 279 DLBCL patients aged 22–90 years (median, 68.0 years) were studied. The follow-up period for the R-CHOP group ranged from 7 to 102 months (median, 25.2 months). Patient and disease characteristics for both treatment cohorts, including the five clinical parameters that comprise the IPI, are listed in Table 1. The case distribution of immunohistochemically defined GCB and non-GCB subtypes is also shown in Table 1. The DLBCL patients (730 cases) were included into 530 (72.6%) nodal lymphoma (49.1% of GCB subtype and 50.9% of non-GCB subtype) and 200 (27.4%) extranodal lymphoma (46.0% GCB subtype and 54% non-GCB subtype). The original sites of lymphoma were 453 lymph node, 62 stomach, 62 Waldeyer's ring, 26 intestine, 22 bone marrow, 15 spleen, 15 testis, 13 skin, 12 nasal cavity, 10 thyroid gland, seven breast, seven eyelid, six parotid gland, and 20 other sites. The non-GCB subtype was much more frequent than the GCB subtype in breast (85.7%), bone marrow (68.2%), and nasal cavity (66.7%).

Table 2. Log-rank analysis of individual immunohistological marker in diffuse large B-cell lymphoma (DLBCL) patients treated with cyclophosphamide, vincristine, doxorubicin, and prednisolone (CHOP) and rituximab (R)-CHOP

Characteristic		CHOP (n = 451)			R-CHOP (n = 279)		
		n (%)	3-year OS (%)	P-value	n (%)	3-year OS (%)	P-value
CD10	Negative	325 (72.1%)	60.5		180 (64.5%)	73.0	
	Positive	126 (27.9%)	70.7	0.022	99 (35.5%)	68.3	0.232
Bcl-6	Negative	240 (53.2%)	58.1		114 (40.9%)	63.0	
	Positive	211(46.8%)	69.4	0.021	165 (59.1%)	77.7	0.018
MUM1	Negative	255 (56.5%)	67.2		139 (49.8%)	69.5	
	Positive	196 (43.5%)	58.5	0.011	140 (50.2%)	74.3	0.752
Bcl-2	Negative	269 (65.9%)	65.9		156 (55.9%)	72.8	
	Positive	182 (40.3%)	59.6	0.001	123 (44.1%)	69.6	0.544
Cell origin	GCB	203 (45.0%)	66.8		130 (46.9%)	68.0	
	non-GCB	248 (55.0%)	59.9	0.050	149 (53.4%)	67.2	0.756

OS, overall survival.

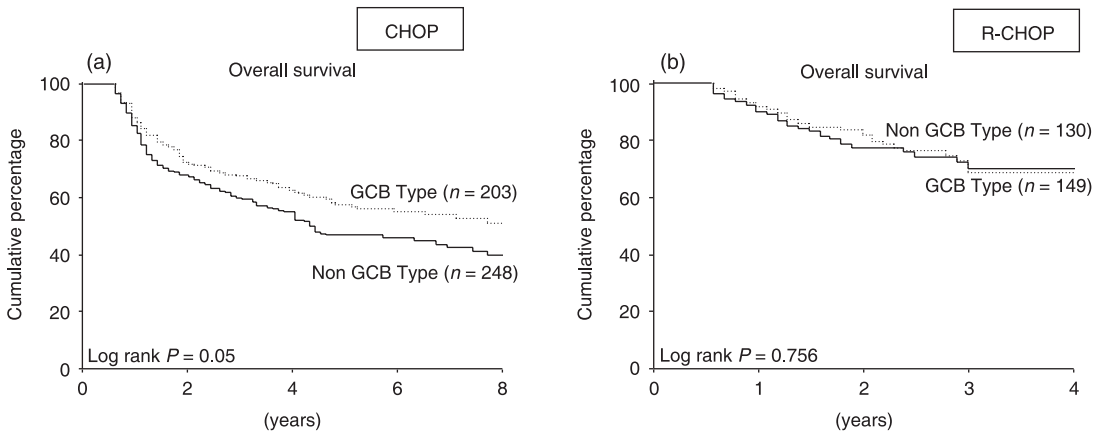

Fig. 1. Overall survival curves of patients with diffuse large B-cell lymphoma of germinal center B (GCB) and non-GCB subtype. Kaplan–Meier curves of overall survival in 451 patients treated with (a) cyclophosphamide, vincristine, doxorubicin, and prednisolone (CHOP) and (b) 279 patients treated with rituximab (R)-CHOP. Classification of GCB and non-GCB subtype was based on the immunohistochemical algorithm.

Survival analyses. The overall response (complete response + complete response unconfirmed)[19] to treatment was 68.5% in the CHOP group and 77.4% in the R-CHOP group ($P = 0.033$). To assess whether the survival of DLBCL patients improved with the addition of R, we compared patient outcomes between the CHOP group and the R-CHOP group. A significant difference in outcome was observed between the two groups. According to the Kaplan–Meier estimates, the OS rates at 36 months were 72.5% in the R-CHOP group and 63.4% in the CHOP group ($P = 0.005$; data not shown). Similarly, the progression-free survival (PFS) rates at 36 months were 61.6 and 51.7% for the R-CHOP and CHOP groups, respectively ($P = 0.0013$; data not shown). These differences were observed in both younger (<60 years) and older (≥60 years) groups and in both high- and low-risk IPI subgroups (data not shown). Therefore, the addition of R to standard chemotherapy showed a proof-of-survival benefit in the present study.

The results of immunohistochemical staining are summarized in Table 2. In the CHOP group, as assessed by the log-rank test, high expression of Bcl-6 and CD10 was associated with a significantly longer OS ($P = 0.022$ and $P = 0.021$, respectively) (Table 2), whereas high expression of Bcl-2 and MUM1 was an adverse predictor of OS ($P = 0.001$ and $P = 0.011$, respectively) (Table 2). However, in the R-CHOP group, CD10, MUM1, and Bcl-2 lost their prognostic value, whereas Bcl-6 remained a significant prognostic marker (Table 2).

Clinical outcomes defined immunohistologically as GCB versus non-GCB subtypes are shown in Figure 1 (pre-R era, Fig. 1a; post-R era, Fig. 1b). The survival rate in the CHOP group was significantly better for the GCB subtype than for the non-GCB subtype ($P = 0.05$) (Table 2; Fig. 1a). However, when the outcomes were compared between the GCB and non-GCB subtypes in the R-CHOP group, no significant differences were found in OS (Table 2; Fig. 1b). These results were similar to those reported previously.[18,23,24]

The clinically based IPI differentiated the four prognostic groups, with 36-month OS ranging from 33.1 to 84.6% ($P < 0.001$) in the CHOP group and from 44.1 to 90.0% in the R-CHOP group ($P < 0.001$; Fig. 2a,b). Although the IPI successfully predicted the survival of patients treated with R-CHOP, the high–intermediate-risk and high-risk groups exhibited closely overlapping curves (Fig. 2b). To study the impact of the revised IPI as a prognostic indicator in the R-CHOP group, we examined the clinical outcome and obtained results similar to those reported by Sehn *et al.*[25] as follows: very good (PFS 82.8%, OS 94.8%), good (PFS 65.8%, OS 83.0%), and poor (PFS 49.3%, OS 51.9%) risk subgroups over 36 months of observation time (PFS, $P < 0.001$; OS, $P < 0.001$; Fig. 2c).

By the Cox proportional hazards regression model, in the CHOP group, high expression of Bcl-2 was associated with a significantly worse survival rate, independently of Bcl-6 and IPI parameters, whereas Bcl-6 were significant prognostic factors independently of Bcl-2 and IPI parameters in the R-CHOP group (Table 3).

In addition, the multivariate analysis data using the IPI scoring system, instead of individual included factors in IPI showed that

doi: 10.1111/j.1349-7006.2009.01268.x

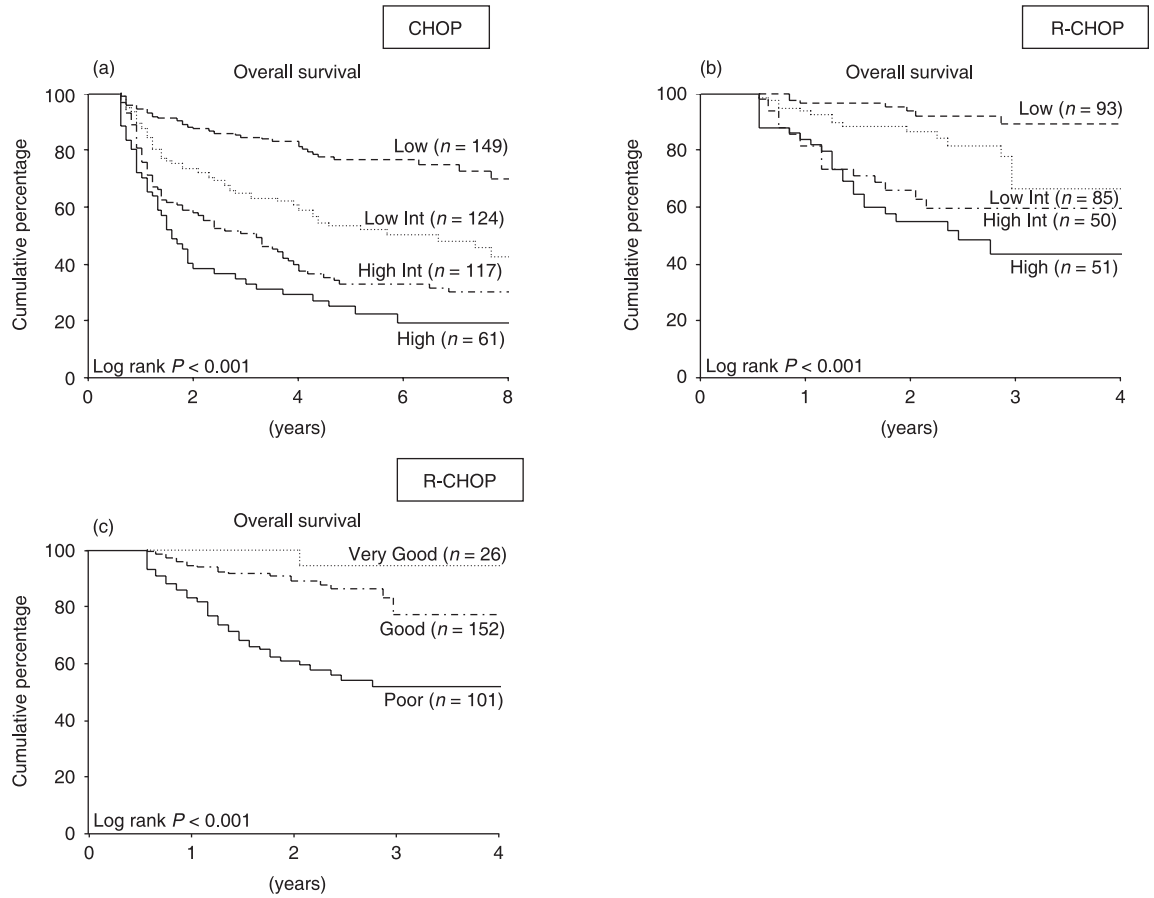

Fig. 2. Overall survival and progression-free survival of patients with diffuse large B-cell lymphoma according to International Prognostic Index (IPI) risk group. Kaplan–Meier curves of overall survival in (a) 451 patients treated with cyclophosphamide, vincristine, doxorubicin, and prednisolone (CHOP) and (b) 279 patients treated with rituximab (R)-CHOP according to the common IPI are shown. (c) In the R-CHOP group, overall survival is shown for each risk subgroup classified by the revised IPI.

Table 3. Cox proportional hazard regression analysis for cyclophosphamide, vincristine, doxorubicin, and prednisolone (CHOP) and rituximab (R)-CHOP

Clinical outcome	CHOP (n = 451)		R-CHOP (n = 279)	
	HR (95% CI)	P-value	HR (95% CI)	P-value
OS Bcl-2 (+ vs –)	1.331 (1.000–1.773)	0.049	1.160 (0.653–2.062)	0.612
Bcl-6 (– vs +)	1.243 (0.896–1.724)	0.194	2.173 (1.116–4.230)	0.022
Age (per year)	1.028 (1.014–1.042)	<0.001	1.032 (1.009–1.055)	0.006
Clinical stage (III–IV vs I–II)	1.940 (1.438–2.617)	<0.001	1.986 (1.031–3.825)	0.040
ECOG PS (II–IV vs 0–I)	1.400 (0.998–1.964)	0.050	2.526 (1.366–4.670)	0.003
Lactate dehydrogenase (>normal)	1.817 (1.350–2.444)	<0.001	2.416 (1.247–4.679)	0.009
No. extranodal sites (≥2 vs 0–1)	1.233 (0.862–1.763)	0.251	0.976 (0.505–1.885)	0.942

Adjusted for age, cell origin, and center effect. CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio.

IPI was a strong independent prognostic factor for OS in both treatment groups (CHOP: hazard ratio for death, 1.565; 95% confidence interval, 1.377–1.788; $P < 0.001$. R-CHOP: hazard ratio for death, 1.746; 95% confidence interval, 1.407–2.168; $P < 0.001$).

Discussion

The aims of the present study were to confirm the survival benefit of R addition and to determine whether previously recognized prognostic factors, based on the classification of DLBCL by cell origin, continue to have prognostic importance in the current R era in Japan.

Consistent with previous reports from Western countries,[4,26] our large-scale study confirmed that the addition of R to standard anthracycline-based CHOP-like regimens provided an additional survival benefit to DLBCL patients in Japan.

We demonstrated that the expression of CD10, BCL-6, MUM1/IRF4, and Bcl-2 proteins predicted survival in the CHOP group, similar to previous reports.[27] The R-CHOP group, in contrast, showed no significant correlation between the

expression of CD10, MUM1/IRF4, and Bcl-2 and patient outcome. However, the expression of Bcl-6 was associated with a superior OS ($P < 0.018$) even in the R-CHOP group.

In the Group d'Etude des Lymphomes de l'Adulte (GELA) trial,[10] no correlation between Bcl-2 overexpression and survival was observed in patients treated with CHOP-R, implying that R addition had overcome the negative effect of Bcl-2. We confirmed this finding in the present study.

As shown in Table 3, we found that Bcl-6 protein was a significant predictor, independent of IPI, in the multivariate analysis in the R-CHOP group. Wilson *et al*. also reported the prognostic value of Bcl-6 expression in DLBCL patients treated with R incorporated into dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin (EPOCH) treatment.[28] However, in the US Intergroup Trial, Bcl-6-positive and Bcl-6-negative patients had similar outcomes when R was incorporated into the treatment regimen.[11] One cause of the difference may be the variation of the cut-off value used among these reports. The percentage of Bcl-6-positive cases (80%) in a previous study[11] was reported to be much higher than that in our present study (59.1%). In addition, the international Lenenburg Lymphoma Biomarker Consortium found a very low reproducibility and unstable staining pattern for Bcl-6 in DLBCL compared to that for other markers.[29,30] Conclusive evidence regarding the significance of Bcl-6 as a predictive marker remains to be resolved.

In the CHOP group, there was a significant difference in the response to treatment between patients with the GCB and non-GCB subtypes ($P = 0.05$). However, in the R-CHOP group, the difference was not observed because of the improvement in the non-GCB subgroup ($P = 0.756$), as shown in Figure 1. Similar data regarding the loss of prognostic value for the distinction of the origin of immunohistochemically defined cells have been reported for patients treated with R-CHOP.[23] Non-GCB (mainly activated B-cell type) lymphoma cells are at least in part regulated by NF-κB systems, leading to upregulation of Bcl-2, an anti-apoptotic protein.[31–33] R is suggested to suppress Bcl-2. Hence, it is likely that patients with the non-GCB subtype would benefit from R addition. In contrast to the results from our present study and previous reports, Fu *et al*. have recently reported that a difference in survival between GCB and non-GCB subtypes was still present in the R era.[34] The reason for this discrepancy is unknown, but it could result from multiple factors. The immunohistochemistry algorithm derived by Hans *et al*. to assign DLBCL to the GCB or non-GCB subtype based on the protein expression of Bcl-6, CD10, and MUM-1[18] is considered imperfect and has a misclassification rate of 19.7% compared to gene expression profiling data.[16]

The multivariate analysis of prognostic factors presented in Table 3 highlights the continued importance of the IPI scoring system. The results of the present study indicate that patients with higher IPI risk scores require a new therapeutic strategy. Attention to both gene and protein expression patterns and IPI scores in the evaluation of novel therapies is therefore justified.

Because our study has the limitations associated with a retrospective analysis, prospective clinical trials with a larger number of patients treated with R plus standard chemotherapy are needed to elucidate reliable molecules that are predictive markers of survival in DLBCL patients.

Acknowledgments

This study was carried out in collaboration with the hematologists and pathologists associated with the Kyushu Lymphoma Study Group. We appreciate all of their efforts and expertise. We thank Konomi Takasu, Toshihiko Murayama, Noburuki Arima, Mayumi Miura, Kohei Yamamoto, Kei Shimizu, Yoshizo Kimura, Kennosuke Karube, Ichio Imamura, Yasuhide Nonaka, Toru Hayashi, Shinji Kouno, Shinichi Aishima, Fumio Yamasaki, Koichi Higaki, and Hiroshi Tanaka for technical assistance. We thank Dr Hironori Koga, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, for helpful discussions.

Abbreviations

CHOP-E	CHOP plus etoposide
EPOCH	etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin
IRF-4	Interferon regulatory factor-4
MACOP-B	methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisolone, and bleomycin
MUM-1	Multiple myeloma-1
THP-COP	cyclophosphamide, therarubicin, vincristine, and prednisolone

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Appendix I

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