original article

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Long-term follow-up results of no initial therapy for ocular adnexal MALT lymphoma

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Background: The majority of lymphomas in the ocular adnexa are low-grade B-cell lymphomas of mucosa-associated lymphoid tissue (MALT lymphoma). Although radiotherapy is the most frequently applied management, cataract and dry eye are problematic complications.

Patients and methods: Between 1973 and 2003, the clinical features of 36 patients with ocular adnexal MALT lymphoma with no symptoms who were managed with no initial therapy after biopsy or surgical resection were retrospectively analyzed.

Results: The median patient age was 63 years (range 22–84) and all patients had stage I disease, consisting of 31 unilateral cases and five bilateral cases. With a median follow-up of 7.1 years, 25 (69%) did not require treatment. The median time until the initiation of treatment in the remaining 11 patients (31%) was 4.8 years. Six patients (17%) died, and among them only two (6%) died due to progressive lymphoma. Seventeen patients (47%) progressed, but histologic transformation was recognized in only one (3%). The estimated overall survival rates of the 36 patients after 5, 10 and 15 years were 94%, 94% and 71%, respectively.

Conclusions: In selected patients with ocular adnexal MALT lymphoma, no initial therapy might be an acceptable approach, because 70% of patients remained untreated at a median of 8.6 years, and their survival was comparable to that of reports on immediate therapy.

Key words: MALT lymphoma, ocular adnexa, no initial therapy, prognosis

introduction

An extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma), first described in 1983 by Isaacson and Wright, was recognized in 1994 as a distinct entity of low-grade B-cell lymphoma in the revised European–American lymphoma (REAL) classification among marginal zone B-cell lymphomas, as well as in the most recent classification of the World Health Organization (WHO) [1–3]. Generally, the majority of MALT lymphomas have an indolent natural history and two-thirds of patients with MALT lymphoma have localized disease [4, 5]. For localized MALT lymphoma, radiotherapy is the most frequently applied management, and most patients show good response to radiotherapy, although several recent reports suggest that radiotherapy alone may not provide for a superior outcome [6–10].

For the management of lymphomas in the ocular adnexa, especially for localized disease, radiotherapy is a safe and effective form of local treatment [11–16]. Histology according to the REAL or WHO classification can be used to accurately predict the prognosis of lymphomas in the ocular adnexa, and the MALT type has a more favorable prognosis than do malignant lymphomas of differing histology [17–20]. Although there have been few analyses of large numbers of MALT lymphomas in the ocular adnexa, its prognosis is thought to be better.

In general, low-grade lymphoma spontaneously regresses on several occasions. Conservative management by deferring treatment until disease progression is an acceptable option for selected patients with follicular lymphoma [21, 22]. This watch-and-wait strategy was initially applied to advanced-stage patients, but a recent retrospective study from Stanford University suggested that this conservative approach is applicable to selected patients with Stage I or II follicular lymphoma [23]. For the radiotherapy of ocular adnexal lymphoma, cataract and dry eye are problematic complications in some patients. From these points, it is controversial whether all patients with MALT lymphoma in the ocular adnexa should be treated with radiotherapy. There is only one report on a small number of patients according to the watch-and-wait approach for conjunctival MALT lymphoma [24]. Therefore, we conducted a retrospective analysis of

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36 patients with early stage disease for whom therapy was deferred after diagnosis at the National Cancer Center Hospital. The objective of this study was to analyze the outcome of the watchful waiting strategy after the diagnosis of early stage MALT lymphoma in the ocular adnexa in view of overall survival, time to treatment, the incidence of and outcome after disease progression, and cause of death.

patients and methods

patient selection

From 1973 to 2003, 36 patients who were diagnosed with primary MALT lymphoma in the ocular adnexa by biopsy or surgical resection were retrospectively analyzed. The criteria used for selection included patients who were diagnosed or reviewed with MALT lymphoma in the ocular adnexa and were managed with no initial therapy after diagnosis and a minimum follow-up of 6 months at the National Cancer Center Hospital, Tokyo, Japan.

The histopathologic diagnosis of MALT lymphoma was established by biopsy or surgical resection of the primary lesion. For all patients, the diagnosis was reviewed according to the REAL or WHO classification by two hematopathologists (Y.M. and A.M.).

clinical features

For patients with a confirmed diagnosis of MALT lymphoma in the ocular adnexa, several sets of clinical data were analyzed based on their medical charts. Sex, age, clinical stages, involved sites, performance status (PS) according to the Eastern Cooperative Oncology Group scale, serum lactate dehydrogenase (LDH) at initial presentation and the degree of surgical resection were analyzed. The anatomically involved sites were determined using the classification of Knowles et al. [25] as previously reported. Physical examinations were performed on all patients. Chest X-ray, computed tomography of the head/eye, neck, chest, abdomen and pelvis, gallium scintigraphy, bone marrow aspiration and peripheral blood examinations were also carried out. The clinical stages were determined according to the Ann Arbor staging classifications.

prognostic factor analysis

All eligible patients were exclusively followed-up at the National Cancer Center Hospital. If patients were lost during follow-up for more than 1 year, we personally contacted them via telephone for information regarding survival, progression and treatment after obtaining their informed consent. Survival was censored at the time of their last documented follow-up date. Overall survival, time to treatment and time to progression or recurrence were calculated from the diagnosis using the Kaplan–Meier method. We evaluated sex, age (<60 versus ≥60), involved site (orbit versus other sites), laterality (unilateral versus bilateral) and the degree of resection (complete resection vesus partial resection or biopsy) using the log rank test. The statistical analysis was performed using SPSS for Windows version 11.0 (SPSS Inc, Chicago, IL).

results

patients' characteristics

From 1973 to 2003, 36 patients who had no symptoms or complaints were diagnosed with primary MALT lymphoma in the ocular adnexa and were managed with no initial therapy after biopsy or surgical resection. Their median time to treatment was 7.1 years, range 0.7–16.7 years. Two cases were identified between 1973 and 1982, 14 between 1983 and 1992, and 20 between 1993 and 2003. The characteristics of the patients are shown in Table 1. Their median age was 62.5 years with a range of 22–84 years, and they consisted of 16 female and 20 male patients. Twenty-one patients (58%) were 60 years of age or older. The most frequently involved site was the orbit at 53%. In 31 patients (86%), their diseases were localized in the unilateral ocular region, whereas five patients (14%) had disease in the bilateral regions. All patients had stage I disease. The number of unfavorable factors according to the international prognostic index (IPI) was one in 19 of 21 patients 60 years or older, and 0 in 15 of 15 patients younger than 60 years, which were consistent with their low risk features.

reasons for no initial therapy

The reasons for giving no initial therapy varied among the patients as shown in Table 2. Seven patients (19%) underwent no initial treatment based on patient preference. These patients had no symptoms such as diplopia, ptosis and swelling. When informed of the therapeutic options, they selected no initial therapy and close follow-up. Twenty-eight patients (78%) had no initial therapy based on physicians' suggestions. The reasons why the physicians recommended no initial therapy varied, including histopathologic diagnoses of low-grade lymphoma, complete surgical resection, and histopathologic diagnoses as benign process or borderline disease such as pseudolymphoma. One patient had no initial therapy because of advanced age.

patient outcome

With a median follow-up of 10.5 years (range 0.7–16.7 years), the estimated median survival was not reached. The proportions of patients who remained alive after 5, 10 and 15 years were 94, 94 and 71%, respectively (Figure 1). There were no differences

Table 1. Patients' characteristics

	No. of patients ($n = 36$)
Sex	
Male	20
Female	16
Age (years)	
Median	62.5
Range	22–84
Involved sites	
Conjunctiva	15
Orbit	19
Lacrimal gland	2
Laterality	
Right	15
Left	16
Bilateral	5
Stage I	36
LDH > normal	2
Degree of resection	
Complete resection	9
Partial resection	24
Biopsy	3

Table 2. Reasons for no initial therapy

	No. of patients (n = 36)
Patient preference	7 (19%)
Physician preference	28 (78%)
Advanced age	1 (3%)

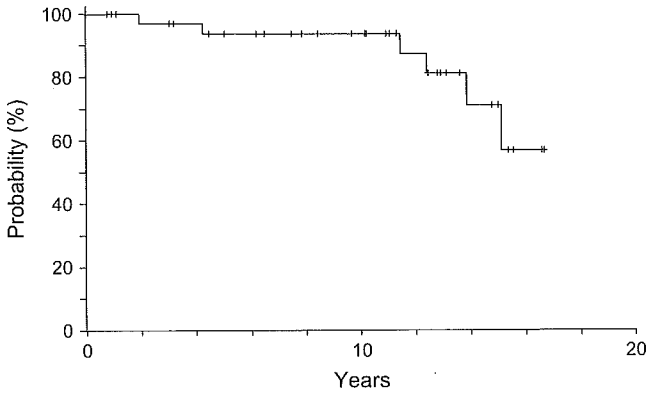

Figure 1. The estimated overall survival of 36 patients with ocular adnexal MALT lymphoma who were managed with no initial therapy. After 5, 10 and 15 years, the probability of overall survival was 94%, 94% and 71% respectively.

in overall survival according to age (<60 years versus ≥60 years), the involved sites and the degree of resection (Figure 2). Six patients died and only two deaths occurred because of progression of their lymphomas. One death was due to heart failure, two were due to pancreatic cancer, and the remaining was due to lung cancer.

Seventeen patients suffered from clinical recurrence or progression. Univariate analysis of patient characteristics and other variables affecting progression and the necessity of subsequent therapy are shown in Table 3. There were no clinical factors that influenced the progression and necessity of subsequent therapy. Only two patients progressed to systemic disease, while 15 showed recurrence or progression as local disease. In six of these 15 patients with local recurrence, further observation without any treatment was selected because of no manifestations of severe clinical symptoms. Only one patient was diagnosed with transformation to a higher-grade lymphoma.

As shown in Figure 3, 25 patients (69%) have not been treated for their diseases to date. At a median follow-up of 7.1 years, more than half have not received treatment for significant disease progression or transformation. The proportions of patients estimated to have not undergone treatment after 5, 10 and 15 years were 80, 63 and 57%, respectively. There were no differences in freedom from requiring treatment according to age (<60 years versus ≥60 years), the involved sites and the degree of resection (Figure 4). Eleven patients (31%) received treatment after recurrence or progression with a median time to treatment of 4.8 years (range 1.0–10.9 years). Nine patients received radiotherapy alone, one received combination radiation therapy and chemotherapy, and one underwent

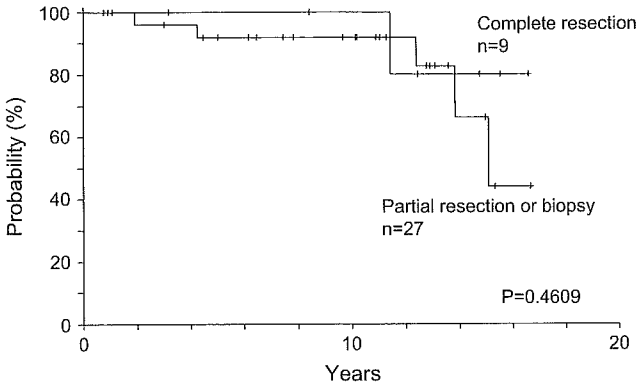

Figure 2. Overall survival according to the degree of resection on initial diagnosis.

Table 3. Univariate analysis of factors affecting progression and necessity of subsequent therapy

Characteristics	Probability (%)			
	5-year progression	P value	5-year subsequent therapy	P value
Sex				
Male	17.4	0.168	17.4	0.397
Female	39.1		14.9	
Age (years)				
<60	30.0	0.938	22.2	0.736
≥60	23.3		11.5	
Involved sites				
Conjunctiva/lacrimal gland	28.6	0.713	13.5	0.635
Orbit	31.0		18.4	
Laterality				
Unilateral	26.8	0.642	15.0	0.580
Bilateral	25.0		25.0	
Degree of resection				
Complete resection	28.6	0.224	0	0.787
Partial resection/biopsy	25.5		16.0	

complete surgical resection. These therapies were effective except for one patient who had progressed to systemic disease.

As initial therapy, we included nine patients who received complete surgical resection alone in the no initial therapy cohort, because it is not standard management for localized lymphoma, although conclusive evidence is lacking in indolent lymphoma, especially in MALT lymphoma. On the other hand, as salve therapy after recurrence or progression, we categorized one patient who received complete surgical resection as the one who required treatment to avoid possible bias leading to our conclusion supporting no initial therapy.

discussion

No initial therapy, often referred to as a watch-and-wait strategy, is recommended as an acceptable treatment option for patients with advanced-stage, low grade B-cell lymphoma,

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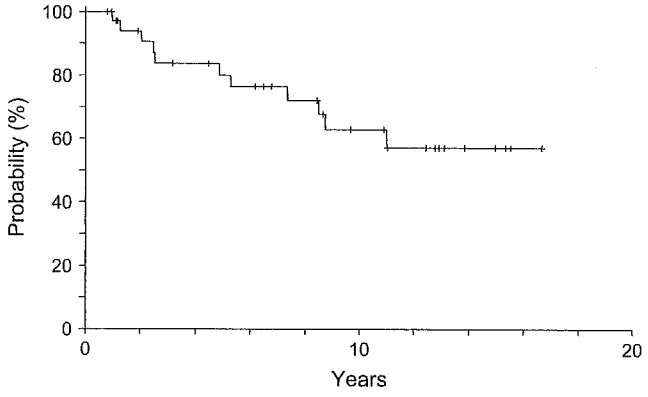

Figure 3. Freedom from requiring treatment. After 5, 10 and 15 years, freedom from requiring treatment was 80%, 63% and 57%, respectively.

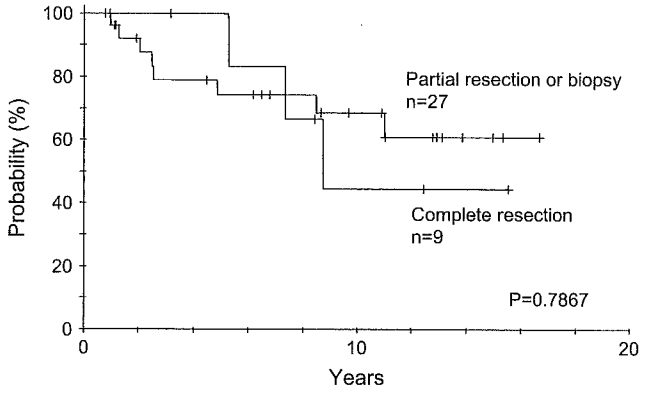

Figure 4. Freedom from requiring treatment according to the degree of resection on initial diagnosis.

especially for those with follicular lymphomas [21, 22]. However, the validity of the watch-and-wait strategy has not been evaluated using a large number of patients with MALT lymphoma. To assess the validity of this approach, we retrospectively analyzed our institutional records for the past 30 years. With a median follow-up of 10.5 years, the estimated overall survival rates after 5, 10 and 15 years were 94, 94 and 71%, respectively. Only 11 patients (31%) required treatment during the follow-up period, with 10 for local recurrence or progression and only one for distant disease. These results suggest that no initial therapy for MALT lymphoma may warrant evaluation.

In our series, six patients died and only two deaths occurred because of progressive disease; one died due to heart failure and two pancreatic cancer, and one died because of lung cancer. The cause of death was not limited to the progression of MALT lymphoma. It has been suggested that even if the start of therapy for MALT lymphoma in the ocular adnexa is delayed, the prognosis might not be worse in selected patients.

No prospective trials have compared immediate radiation or chemotherapy with no initial therapy for patients with MALT lymphoma in the ocular adnexa. To our knowledge, this report is the first to analyze deferred therapy for a large number of patients with stage I MALT lymphoma in the ocular adnexa. Matsuo and Yoshino [24] reported on the follow-up results of observation or radiation for 13 patients with conjunctival malignant lymphomas. At a mean follow-up of 5.4 years, seven of eight patients under observation experienced spontaneous regression. Their conclusions were similar to ours where no initial therapy might be an acceptable therapeutic option. However, the patient population that was studied in their report was distinct from ours, because the initial site was limited to the conjunctiva in their study. In our analysis, the lymphomatous lesions were not limited to the conjunctiva, and there was no difference in patient outcome according to the initial site. In addition, the degree of surgical resection did not influence patient outcome as shown in Figures 2 and 4. These results suggest that no initial therapy is applicable to selected patients with ocular adnexal MALT lymphoma regardless of the degree of surgical resection.

Table 4 shows a comparison of the overall survival of patients with ocular adnexal lymphoma treated with immediate radiotherapy at several institutions and those of the present study who were managed with no initial therapy. Two previous reports focused on radiation for patients with MALT lymphoma [13, 16]. Table 4 reveals that the estimated overall survival rates in our series were not inferior to those of patients treated with immediate radiotherapy reported by other institutions, although the patient selection criteria might have differed among these studies.

Primary radiotherapy for localized MALT lymphoma in the ocular adnexa offers excellent local control with a prolonged clinical course [11–16]. However, recurrent disease can occur at local initial and distant sites. Uno et al. [16] described that responses to radiotherapy included a complete response in 26 of 50 patients, a partial response in 20 patients and no change in four patients. None of the patients who achieved a complete response experienced local recurrence, and five of the 20 who achieved a partial response exhibited recurrent disease 4–97 months after radiotherapy. Le et al. [13] indicated that local control by radiotherapy is 100% effective and that most of the failures arise in extranodal sites. In our series, there were 17 of 36 (47%) patients with clinical recurrence or progression. Among them, only two progressed to systemic disease, whereas 15 remained with local disease. In six of the 15 patients with local recurrence, further observation was selected because of the lack of manifestations of severe clinical symptoms. Only one patient showed histologic transformation to a higher-grade lymphoma. Although primary radiotherapy for localized MALT lymphoma in the ocular adnexa offers better local control than does no initial therapy, recurrence could have taken place in the immediate local radiotherapy group and no initial therapy group. There were few transformations to aggressive lymphoma in both groups, and there was probably no difference in the overall survival between these two therapeutic strategies as shown in Table 4. Considering these findings, no initial therapy may be an acceptable option in a fraction of patients with ocular adnexal MALT lymphoma.

In the present study, 25 of 36 patients (69%) have not been treated for their disease to date. At a median follow-up of 7.1 years, more than half of the patients had not received treatment for significant disease progression or transformation. This is notable because quality of life is an important issue for patients who wish to avoid early and late therapy-related complications.

Table 4. Overall survival in ocular adnexal lymphoma: comparison with other studies by immediate radiotherapy

Author	No. of patients	Histology	Stage	Median age (years)	5-year, 10-year survival (%)
Uno et al.	50	MALT	I	61	91, 76
Le et al.	31	MALT	I	54	100, 73
Bhatia et al.	47	Low/intermediate	I	69	74, NA
Martinet et al.	90	Low/intermediate/high	I–II	63.5	78, 70
Stafford et al.	48	Low/intermediate	I–IV	68	69, 40
Present study	36	MALT	I	62.5	94, 94

Recently, Conconi et al. [26] revealed that rituximab has clinical activity against MALT lymphomas. Also, Ferreri et al. reported that *Chlamydia psittaci*-eradicating antibiotics therapy resulted in an objective response in ocular adnexal MALT lymphoma [27, 28]. These therapeutic strategies would yield not only an effective response but also less therapy-related complications. It is clear that most MALT lymphomas in the ocular adnexa are indolent diseases. We believe that no initial therapy, rituximab and antibiotic therapy may be acceptable options for ocular adnexal MALT lymphoma.

More than half of the patients in our series have not yet been treated. These patients clearly benefited from a conservative management approach, although a minority of the patients showed a less favorable disease course. Clinical studies on no initial therapy for localized ocular adnexal MALT lymphoma are limited, and the reasons why most of the patients did not progress for a long period are unclear. Table 3 shows that no clinical factors influenced the time to progression and the necessity of subsequent therapy. Recently, cytogenetic studies have revealed that patients with MALT lymphoma have multiple karyotypic abnormalities, $t(14;18)$ or $t(11;18)$ translocation at diagnosis, and that these karyotypic abnormalities are useful for predicting the response to therapy and their prognosis [6, 29, 30]. There are no reports of cytogenetic or molecular genetic studies in relation to the prognosis of patients with ocular adnexal MALT lymphoma. If prediction of the clinical outcome of each patient is possible, more suitable therapeutic decisions including no initial therapy for each patient may be applicable.

In conclusion, survival in selected patients with localized MALT lymphoma in the ocular adnexa does not appear to be worse when no initial therapy is substituted for local radiotherapy. With the prediction of long median survival, quality of life is an important issue for patients who wish to avoid early and late therapy-related complications. Extended follow-up will be required to assess the impact of no initial therapy on overall survival and freedom from requiring treatment. Although the most frequently applied management of localized MALT lymphoma in the ocular adnexa is radiotherapy, we believe that no initial therapy is an acceptable option for selected patients.

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Follicular lymphoma subgrouping by fluorescence *in situ* hybridization analysis

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The frequency of t(14;18) in follicular lymphoma (FL) in Japan has been reported to be low compared to North America and other European countries. Recently, it has also been reported that FL lacks t(14;18), mainly among histological grade 3b, and occasionally has a rearranged *Bcl-6* gene. It is not known whether a difference in histology or immunostaining pattern exists between FL with and without t(14;18). We performed interphase fluorescence *in situ* hybridization (FISH) analysis to detect *Bcl-2/IgH*, *Bcl-6* gene rearrangement, *Bcl-2* gene amplification, and the *cyclinD1/IgH* gene in formalin-fixed paraffin embedded specimens from our FL archives. The correlation between morphological features, histological grades, immunohistochemical findings, and cytogenetical aberrations was studied. In total, we found that 28 of 47 cases (59.6%) had t(14;18). *Bcl-6* gene rearrangement and extra *Bcl-2* gene signals were found in five and two cases, respectively. Only one had *cyclinD1/IgH* fusion. Ten of 12 grade 1, nine of 17 grade 2, and 0 of two grade 3 cases had fusion signals, respectively. None of the above abnormalities were detected in 12 of 47 cases (25.5%). Our data confirmed a high frequency of t(14;18) in FL in grade 1, but a lower incidence among grade 2, that could be attributed to the lower incidence of the translocation in FL in Japan. Immunostaining of both *Bcl-2* and CD10 was highly predictable for the presence of t(14;18); the positive predictive value was 75%, suggesting the usefulness of the staining. (*Cancer Sci* 2005; 96: 77–82)

Follicular lymphoma (FL) is derived from follicular center B-cells and morphologically has a predominantly follicular pattern.[1–3] The neoplastic cells are commonly positive for CD10 and Bcl-2 protein. The natural history of FL is characterized by occasional transformation to aggressive large B-cell lymphoma after indolent behavior. The translocation (14;18)(q32;q21) has been recognized as a hallmark of FL, and was detected in approximately 80–100% of FL cases in North America and European countries.[4,5] However, in South-east Asia, including Japan, the incidence of FL is low among non-Hodgkin's lymphoma (NHL) cases,[6–9] and moreover, the typical cytogenetic abnormality has been considered relatively low,[5,10–12] suggesting the geographic heterogeneity of this type of lymphoma.

Recently, it has been reported that FL without t(14;18), including cases with *Bcl-6* gene rearrangements and other cytogenetic abnormalities such as trisomy 18, is detected mainly in histological grade 3b in North America and European countries.[13–16] However, the relationship between the chromosomal aberration and morphology, histological grade, and immunohistochemistry has not yet been clarified. We preformed interphase fluorescence *in situ* hybridization (FISH) analysis to detect *Bcl-2/IgH*, *Bcl-6* gene rearrangement, *Bcl-2* gene amplification, and the *cyclinD1/IgH* gene in formalin-fixed paraffin embedded specimens from our FL archives. We also characterized Japanese FL with and without t(14;18) in terms of morphological features, histological grades, and immunohistochemical findings.

Patients and Methods

Patient samples. Cases diagnosed as having FL between December 1998 and December 2003 were selected from the database of the National Cancer Center Hospital (NCCH), Japan. Only cases with complete records of CD10 and Bcl-2 staining by immunohistochemistry were included in this study.

Samples for FISH analysis were obtained from lymph nodes, the gastrointestinal tract, nasopharyngeal area, gallbladder, and bone in 37, 4, 4, 1, and 1 case, respectively, and selected from archives of formalin-fixed paraffin embedded specimens kept at the NCCH.

Histology. Each specimen was stained with hematoxylin and eosin. We categorized three groups by morphological findings: (i) typical types with a lymphoid cuff which indicated a mantle zone, and a diffuse area less than 50%; (ii) a reverse variant which lacked a lymphoid cuff;[17] and (iii) others. Others were defined as none of the above and included those samples in which the diffuse area was more than 50%. Histological grading was also determined according to the World Health Organization classification.[1] Cases with a diffuse large B-cell lymphoma (DLBCL) component in addition to FL were also included. In this study, histological transformation to large B-cell lymphoma was defined as follows: (i) cases with prior biopsy specimens confirmed as FL, or (ii) cases with multiple specimens showing a diagnosis of both FL and DLBCL.

Immunohistochemistry. Immunohistochemichal studies were performed on formalin-fixed paraffin embedded specimens by the standard avidin–biotin methods. Monoclonal antibodies used were for: CD20 (Dako, Glostrup, Denmark), CD3 (Novocastra, Newcastle-upon-Tyne, UK), Bcl-2 (Dako), and CD10 (Novocastra). CD5 (Novocastra), cyclin D1 (Dako), and CD21 (Dako) were added, if necessary.

Preparation of glass slides for FISH analysis. The established tissue FISH procedure was performed with minor modifications of parameters as follows. Each specimen was cut at a thickness of approximately 4 μm. The sample was treated in a solution of 0.3% pepsin/0.01 N HCL for 14 min at 37°C, denatured in 70% formamide/20 × SSC for 2 min at 73°C. A microwave treatment procedure was then carried out to intensify the signals. The microwave (MI-77, Azumaya Company, Tokyo, Japan) was set to beam irradiation at intervals of 3 s on and 2 s off, at a frequency of 2.45 GHz at 250 W output power, with the temperature sensor set to 37°C for 60 min.[18] Incubation was performed overnight at 37°C. Slides were covered with an antifade solution and viewed under a BX60 fluorescence microscope (Olympus, Tokyo, Japan) using a ×100 oil immersion lens and appropriate filters.

We used the reactive lymph node as a negative control and the threshold was determined. The lymph nodes were obtained from surgically resected specimens of solid cancer and had been confirmed to be negative for the cancer cells. If the frequency

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was above this level, the sample was judged to be positive for the aberration. Each specimen was overviewed with low magnification and only cells located on the center of the follicle were evaluated. Each specimen was examined independently by three investigators (Y K, Y Y, N S), and when the judgment was the same, the result was counted as successful staining and included in this study.

Probes applied to FISH analysis. All the probes were purchased from the manufacturer (Vysis, Downers Grove, IL, USA). To detect t(14;18), we used the LSI IGH/BCL2 Dual Color, Dual Fusion Translocation Probe. To detect *Bcl-6* gene rearrangement, the LSI BCL6 Dual Color, Break Apart Rearrangement Probe was used. To detect amplification or deletion of chromosome 18, the CEP 18 Spectrum Orange probe, which hybridizes to the centromeric region of chromosome 18, was used. To detect t(11;14), we used the LSI IGH/CCND1 Dual Color, Dual Fusion Translocation Probe.

First, all the specimens were analyzed by both probes to detect t(14;18) and *Bcl-6* gene rearrangements (Fig. 1a,b). If extra *Bcl-2* signals were detected (Fig. 1c), the CEP18 Spectrum Orange probe was applied to confirm if the extra signals were due to either gaining chromosome 18 or amplification of *Bcl-2* gene/*Bcl-2* gene rearrangement with other genes. Finally, samples without abnormalities following these procedures underwent FISH using LSI IGH/CCND1 Dual Color, Dual Fusion Translocation Probe to detect t(11;14).

Statistical analysis. To compare incidence of t(14;18) among each histologic grade group, we performed chi-squared independence test using a 4×2 contingency table. Because almost all cases with FL + DLBCL had grade 3 FL, we analyzed grade 3a, grade 3b, and FL + DLBCL together as a grade 3 group in the analysis. After the analysis, we performed Fisher's exact probability test to compare the incidence of t(14;18) in each histologic grade.

To test whether or not immunohistochemical staining could predict the presence of t(14;18), we performed sensitivity and specificity tests. A true positive (TP) was defined as being positive for immunohistochemical results in t(14;18)-positive patients, whereas a false-positive (FP) was defined as being negative for immunohistochemical results in t(14;18)-positive patients. A true negative (TN) was defined as being negative for immunohistochemical results in t(14;18)-negative patients, whereas a false-negative (FN) was defined as being positive for immunohistochemical results in t(14;18)-negative patients.

The sensitivity was calculated by TP/(TP + FN), whereas the specificity was calculated by TN/(FP + TN). The positive predictive value was found by TP/(TP + FP) and the efficacy was found by (TP + TN)/total.

Results

Determination of the threshold. Reactive lymph nodes (five samples) were used as negative controls. The thresholds of fused signal frequencies were determined as the mean plus 3 standard deviations (SD) of the number of overlapping or touching signals from 100 to 200 nuclei on each slide (Table 1). 'One fusion signal pattern' was defined by observation of one Orange (O), one Green (G), and 'one Orange/Green (yellow) fusion (F) signal' (1O1G1F pattern) was commonly observed in paraffin-embedded tissue specimens in addition to the theoretical fusion pattern (1O1G2F pattern). We evaluated the frequency of one fusion signal pattern and the threshold was also determined. The calculated thresholds of the IGH/BCL2 2 fusion pattern and 1 fusion pattern were 2% and 7%, respectively (Table 1). If the fusion signals were greater than these thresholds, the samples were judged to be positive for fusion. In the same manner, the cases were judged to have an extra number of *Bcl-2* signals if the frequency was more than 1.5%. The

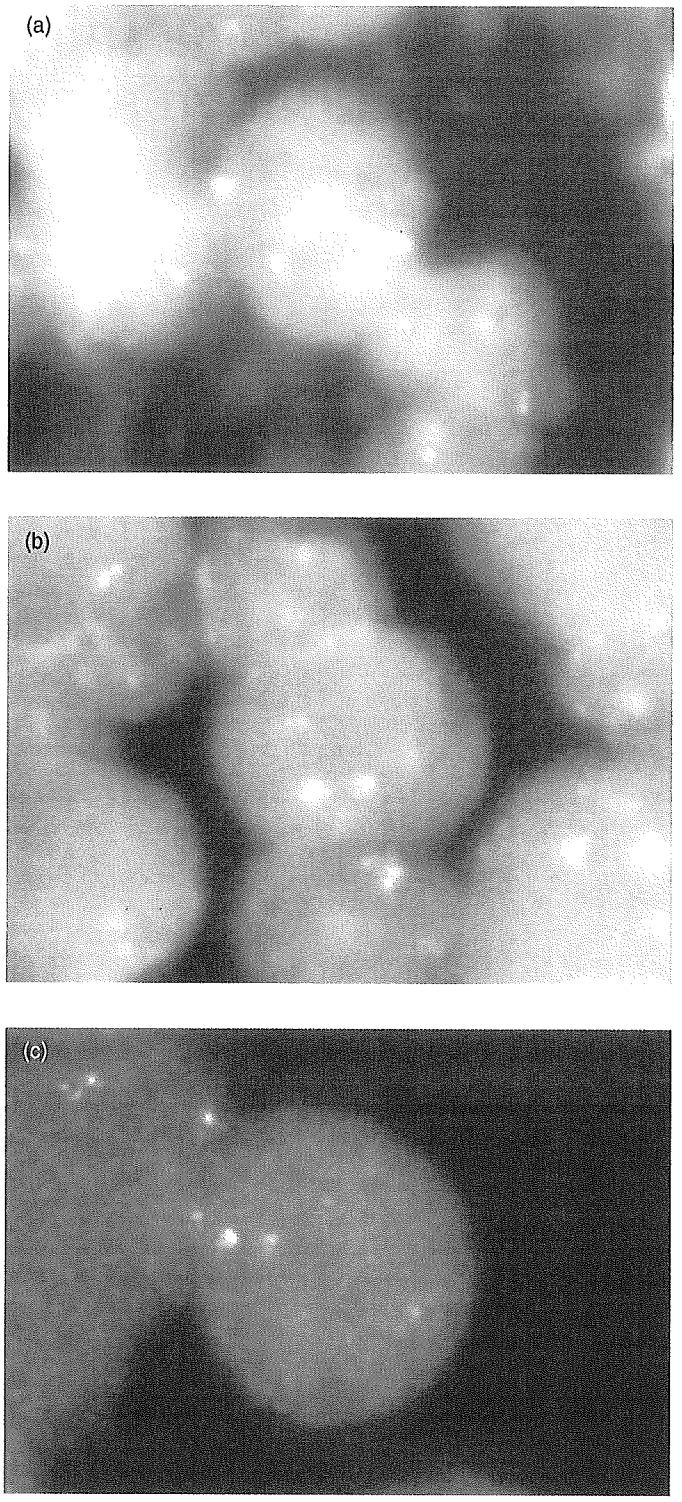

Fig. 1. Representative result of fluorescence in situ hybridization analysis. (a) IgH and Bcl-2 gene fusion pattern with the LSI IGH/BCL2 Dual Color, Dual Fusion Translocation Probe (Vysis, Downers Grove, IL, USA). One orange (1O) Bcl-2 signal, one green (1G) IgH signal, and two fusion (2F) IgH/Bcl-2 signals are present. (b) Bcl-2 gene amplification or rearrangement with other genes with LSI IGH/BCL2 Dual Color, Dual Fusion Translocation Probe. 3O 2G signals are seen. (c) Bcl-6 gene rearrangement with LSI BCL6 Dual Color, Break Apart Rearrangement Probe. 1O 1G 1F signals are shown.

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Table 1. Control of the IGH/BCL2 Dual Color, Dual Fusion Translocation Probe (Vysis, Downers Grove, IL, USA) for reactive lymph nodes

Pattern	Mean (%)	SD	Mean + 3SD (%)	Threshold (%)
1O 1G 2F	0.2	0.48	1.54	2
1O 1G 1F	3.6	0.9	6.28	7
3O 2G or 2O 3G	0	0	0	1.5
1O 2G	17.2	5.7	NA	NA
2O 1G	15.2	4.7	NA	NA
2O 2G	62	12.8	NA	NA

SD, standard deviation; O, orange; G, green; F, fusion; NA, not applicable.

thresholds to 2F and 1F were over 1% and 6%, respectively, when IgH/CCND1 dual color, dual fusion translocation probes were used. The breaking apart of the signal threshold was 1.5% and was again calculated as the mean plus 3 SD of the frequency of break apart signals for the analysis using BCL6 dual color, break apart rearrangement probes.

Results of FISH analysis and immunohistochemistry. In total, we studied samples from 47 cases of FL (Table 2). Among them, two cases were not applicable for CD10 because of insufficient staining for evaluation. Excluding these cases, 41 were positive for CD10 (91.1%) with immunohistochemistry.

Forty-two of 47 cases (89.4%) were positive for Bcl-2 by immunostaining. Twenty-eight of 47 cases (59.6%) were positive for t(14;18) by FISH analysis; one of these 28 patients was also positive for *Bcl-6* gene rearrangement by FISH analysis.

Nineteen cases (40.4%) were negative for t(14;18). Among them, four cases (8.5% of total 47 cases) were positive for *Bcl-6* gene rearrangement, and two cases (4.3%) were accompanied by amplification of the *Bcl-2* gene or *Bcl-2* rearrangement with other genes. One case (2.1%) was shown to have t(11;14); the case was negative for CD5 and cyclin-D1 by immunohistochemistry and re-examination with a microscope showed again that the case had morphological features of FL without evidence of mantle cell lymphoma. Twelve patients (25.5%) had none of the above chromosomal aberrations as shown by FISH (Fig. 2).

Relationship among morphological features, histological grades, and chromosomal aberrations by FISH analysis. The relationship between morphological features and presence of t(14;18), by FISH analysis, was analyzed in the 41 cases without transformation to DLBCL. A total 23 cases had t(14;18) typical histology and among them nine cases showed typical histology, while 18 cases did not have t(14;18), of which six cases showed typical histology. A reverse variant was recognized in four cases with t(14;18), but in no cases without t(14;18). The difference was not significant (Table 3).

The relationship between histological grades and chromosomal aberrations by FISH analysis is shown in Table 4. Ten of 12 cases (83.3%) were positive for t(14;18) in grade 1, and nine of 17 in grade 2 (52.9%), 0 of two in grade 3a (0%), and four of 10 in the FL + DLBCL (40%) groups. However, a relatively high incidence was observed in the histological transformation group at diagnosis (five of six cases, 83.3%).

The incidence of t(14;18) among each histologic grade group was statistically different ($P = 0.047$). The incidence of t(14;18) in grade 2 tended to be lower, and statistically significantly lower in the grade 3 group compared to that in grade 1; the *P*-values were 0.096, and 0.018, respectively.

Extra numbers of *Bcl-2* gene signals were detected both in grades 1 and 2. *Bcl-6* gene rearrangement was recognized in the grade 1, 2, and FL + DLBCL groups; we found five cases had rearrangements. The case judged to have t(11; 14) was grade 1.

Relationship between immunohistochemistry and t(14;18) by FISH analysis. The relationship between immunohistochemistry and t(14;18) by FISH analysis is shown in Table 5. Sensitivity was 100% in all groups, although specificity was 22.2%, 26.3%, and 50.0% in the CD10, Bcl-2, and CD10/Bcl-2-positive groups, respectively. In both the CD10 and Bcl-2 positive groups, the positive predictive value and efficacy were 68.9% and 80.0%, respectively.

Discussion

We found 59.6% of FL cases had t(14;18), in the present study. The t(14;18) has been recognized as a hallmark of FL and it is detected in approximately 90% of FL cases in North America and European countries, whereas the incidence of t(14;18) in East Asia, including Japan, has been reported to be relatively lower than that of North America and European countries.[5,10–12] Amakawa *et al.* reported that only 10 of 30 cases (33.3%) had t(14;18),[10] while Yabumoto *et al.* also reported 45 of 79 cases (57.0%) had the abnormality by Southern blot hybridization.[11] Mitani *et al.* reported 13 of 41 cases (31.7%) had the typical aberration by polymerase chain reaction (PCR) analysis.[12] The higher incidence that we observed might be attributed to the methodology. Conventional cytogenetic analysis commonly fails to detect cytogetetic aberrations, especially in lymph node specimens with low grade lymphoma, including FL. PCR analysis still fails to detect translocation breakpoints outside major breakpoint and minor cluster regions. Therefore, FISH analysis can overcome these weak points and detects a greater number of positive cases. In the present study, using this method, however, the incidence of t(14;18) in Japan was still lower. This is consistent with the recent report by Matumoto *et al.*, which showed 56% of Japanese FL had the translocation using the same procedure.[19]

It has also been reported that FL lacks t(14;18) mainly among histological grade 3b, and that it occasionally has a rearranged *Bcl-6* gene in North America and European countries.[13,14] Ott *et al.* reported that grade 1, 2, and 3a had typical t(14;18) in 61 of 73 cases (84%), whereas two of 16 cases (13%) had t(14;18), and seven of 16 cases (44%) had a chromosomal break at 3q27 in G3b ± DLBCL.[13] Bosga-Bouwer *et al.* reported similar findings by using PCR, and FISH analysis,[14] they found that 10 of 32 cases of G3b without t(14;18) had a 3q27 aberration. In the present study, most grade 1 cases had t(14;18), whereas the incidence of the aberration was relatively low in grade 2, 3a, and FL + DLBCL. Especially in grade 2, only nine of 17 cases were positive for t(14;18), which would lower the total incidence of positive cases among Japanese FL.

We also checked the incidence of the *Bcl-6* gene rearrangement, anticipating a high incidence of this rearrangement among cases without t(14;18); however, that was not the case and the incidence was equal to that of North America and other European countries and was recognized regardless of histological grade. *Bcl-2* gene amplification was also detected in a limited numbers of cases. None of the abnormalities were detected in one-quarter of the cases using our procedure.

We found that FL that transformed to aggressive large B-cell lymphoma had a high frequency of t(14;18). We included these cases, because the clinical course was that of typical FL. The high frequency might reflect the selection bias that observations of a typical indolent clinical course per se reinforce the diagnosis of FL.

We also examined the relationship among morphological features, immunohistochemistry and t(14;18). In the present study, typical morphological features did not indicate the presence of t(14;18), while the reverse pattern did not exclude the absence of t(14;18), either. However, when immunostaining of both Bcl-2 and CD10 were positive, the presence of t(14;18) was highly predictable. This is consistent with Godon *et al.*, who reported that t(14;18) was detected in all 63 cases (100%) with CD10 and Bcl-2-positive FL using FISH analysis.[4] The importance of immunohistchemistry should be stressed when diagnosing FL.

Table 2. Results of clinical data, morphological feature, histologic grade, and fluorescense *in situ* hybridization

Case no.	Sex	Age	Biopsy sites	Morphological feature	Histologic grade	CD10	Bcl-2	t(14;18)	*Bcl-6* B.A.	Extra *Bcl-2*	t(11;14)
1	F	60	Mesentric LN, ileum	Transformed FL	transformed FL	−	+	−	−	−	−
2	F	72	Cervical LN	Typical	G2	+	−	−	−	−	+
3	F	61	Cervical LN, stomach	Transformed FL	transformed FL	+	+	+	−	−	N.D.
4	F	66	Inguinal LN	Others	G2	+	−	−	−	−	N.D.
5	M	59	Inguinal LN	Others	G3a + DLBCL	+	+	−	+	−	N.D.
6	F	30	Inguinal LN	Typical	G3a	+	+	−	−	−	−
7	M	67	Mediastinal LN	Others	G1	+	+	+	−	−	N.D.
8	F	54	Stomach, ileum	Transformed FL	transformed FL	+	+	+	−	−	N.D.
9	F	78	Tonsil, stomach	Reverse	G1	+	+	+	−	−	N.D.
10	M	44	Cervical LN, iliac LN	Transformed FL	transformed FL	+	+	+	+	−	N.D.
11	F	65	Inguinal LN	Others	G3b + DLBCL	−	+	−	−	−	−
12	M	19	Inguinal LN	Typical	G3a + DLBCL	+	−	−	−	−	−
13	F	50	Cervical LN, inguinal LN	Others	G2	+	+	+	−	−	N.D.
14	M	61	Inguinal LN	Reverse	G1	+	+	+	−	−	N.D.
15	M	63	Ilium	Typical	G2 + DLBCL	+	+	+	−	−	N.D.
16	M	74	Tongue, gingiva	Transformed FL	transformed FL	+	+	+	−	−	N.D.
17	M	48	Inguinal LN	Reverse	G1	+	+	+	−	−	N.D.
18	F	44	Mesentric LN, stomach	Others	G1	+	+	+	−	−	N.D.
19	F	62	Cervical LN, stomach	Typical	G2	+	+	+	−	−	N.D.
20	F	55	Axillary LN, stomach	Typical	G1	+	+	+	−	−	N.D.
21	F	38	Inguinal LN	Others	G2	+	+	−	+	−	N.D.
22	M	59	Cervical LN	Reverse	G2	+	+	+	−	−	N.D.
23	F	60	Axillary LN	Others	G2	+	+	−	−	−	−
24	F	72	Inguinal LN	Typical	G2	+	+	−	−	−	−
25	F	54	Mesetric LN	Typical	G1	+	+	+	−	−	N.D.
26	F	45	Inguinal LN, mesentric LN	Others	G2	+	+	+	−	−	N.D.
27	M	70	Inguinal LN	Typical	G2	+	+	−	−	+	N.D.
28	F	32	Mesentric LN	Typical	G1	+	+	+	−	−	N.D.
29	F	67	Cervical LN	Others	G2	+	−	−	−	−	−
30	M	68	Axillary LN	Typical	G3a + DLBCL	N.A.	+	−	+	−	N.D.
31	F	52	Mesentric LN	Others	G1	+	+	+	−	−	N.D.
32	F	42	Axillary LN	Others	G3a + DLBCL	+	+	+	−	−	N.D.
33	M	55	Inguinal LN	Typical	G1	+	+	+	−	−	−
34	M	71	Subcutaneous LN	Others	G3a + DLBCL	−	+	−	−	−	−
35	M	52	Cervical LN, stomach	Others	G2	N.A.	+	+	−	−	N.D.
36	M	49	Inguinal LN	Others	G2	+	+	−	−	−	−
37	M	58	Inguinal LN	Others	G1	−	+	−	−	+	−
38	M	54	Supraclavicular LN	Others	G3a	+	−	−	−	−	−
39	M	54	Cervical LN	Others	G2	+	+	+	−	−	N.D.
40	F	46	Inguinal LN, bladder	Transformed FL	transformed FL	+	+	+	−	−	N.D.
41	F	69	Inguinal LN	Typical	G2	+	+	+	−	−	N.D.
42	F	51	Inguinal LN	Others	G2	+	+	+	−	−	N.D.
43	M	54	Inguinal LN	Others	G3b + DLBCL	+	+	+	−	−	N.D.
44	F	57	Inguinal LN	Others	G1	+	+	−	+	−	N.D.
45	M	56	Pharynx	Others	G3b + DLBCL	+	+	−	−	−	−
46	F	62	Duodenum	Typical	G3b + DLBCL	+	+	+	−	−	N.D.
47	F	55	Cervical LN	Typical	G2	+	+	+	−	−	N.D.

Bcl-6 B.A., Bcl-6 break apart; DLBCL, diffuse large B-cell lymphoma; Extra Bcl-2, Bcl-2 gene amplification or rearrangement with other genes; F, female; FL, follicular lymphoma; G, grade; LN, lymphnode; M, male; N.A., not applicabable; N.D., not done; others, none of the above; reverse, reverse variant; typical, typical histology.

Table 3. Relationship between morphological features and follicular lymphoma with or without t(14;18)

Morphological feature	Follicular lymphoma with t(14;18)	Follicular lymphoma without t(14;18)
Typical histology	9	6
Reverse variant	4	0
None of the above	10	12
Total	23	18

In contrast, FL without t(14;18) tended to lack Bcl-2 protein expression. Horseman *et al.* reported 50 cases of FL without t(14;18) by conventional cytogenetic analysis, and they found only 16 Bcl-2 positive cases among them.[16] In their report, 11 of the 16 cases had a chromosome 18 aberration, including trisomy 18, and only one case had the 18q21 aberration of 34 cases without the Bcl-2 protein. In the present study, we detected two cases with *Bcl-2* gene amplification or rearrangement with other genes and both cases were positive for Bcl-2 protein, which is consistent with their report, suggesting that that Bcl-2 over-expression might be due to an amplified *Bcl-2* gene.

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Table 4. Relationship between histologic grade or group, immunohistochemistry and karyotypic abberation by fluorescense *in situ* hybridization analysis

Histologic grade	CD10(+)	Bcl-2(+)	t(14;18)(+)	t(14;18)(+) Bcl-6(–)	t(14;18)(+) Bcl-6(–)	t(14;18)(+) Bcl-6(+)	Extra Bcl-2	t(11;14)(+)	Total
G1	11	12	10	10	1	0	1	0	12
G2	16 (N.A.1)	14	9	9	1	0	1	1	17
G3a	2	1	0	0	0	0	0	0	2
G3b	0	0	0	0	0	0	0	0	0
FL + DLBCL	7 (N.A.1)	9	4	4	2	0	0	0	10
Transformed FL	5	6	5	4	0	1	0	0	6
Total	41(N.A.2)	42	28	27	4	1	2	1	47

DLBCL, diffuse large B-cell lymphoma; Extra Bcl-2, Bcl-2 gene amplification or rearrangement with other genes; FL, follicular lymphoma G, grade; N.A., not applicabable.

Table 5. Relationship between immunohistochemistry and t(14;18) by fluorescense *in situ* hybridization analysis

	Sensitivity (%)†	Specificity (%)†	Positive predictive value (%)†	Efficacy (%)†
CD10	100	22.2	65.9	68.9
Bcl-2	100	26.3	66.6	70.2
CD10/Bcl-2	100	50.0	75.0	80.0

†Sensitivity, specificity, positive predictive value and efficacy were calculated as TP/(TP+FN), TN/(FP+TN), TP/(TP+FP), and (TP+TN)/total, where TP is true positive, FP is false positive, TN is true negative, and FN is false negative, respectively.

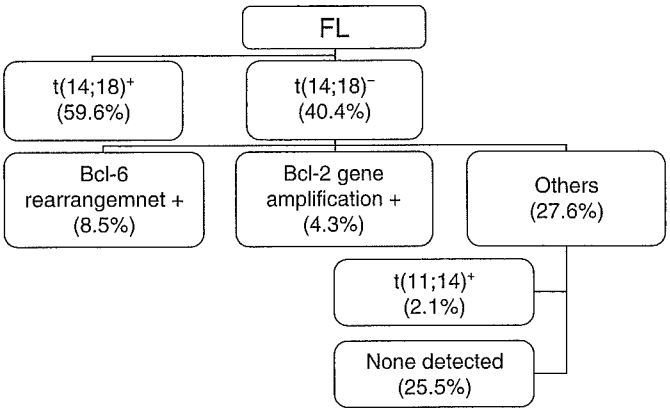

Fig. 2. Results of follicular lymphoma subgrouping by fluorescence in situ hybridization analysis.

One of the other purposes of the present study was to examine the impact of FISH analysis on the pathological diagnosis. The Non-Hodgkin's Lymphoma Classification Project reported that information from immunophenotypic analyses and cytogenetics did not contribute to the accurate diagnosis of FL, although its importance was pointed out for mantle cell lymphoma (MCL).[2,3] We sometimes encounter difficulty distinguishing MCL from FL by pathological diagnosis only. Therefore, we added the LSI IGH/CCND1 Dual Color, Dual Fusion Translocation Probe to detect t(11;14) in patients without any abnormality following an earlier procedure. In the present study, t(11;14) was detected in one case; this patient was negative for CD5 and cyclin D1 by immunohistochemistry. Rosenwald *et al.* identified MCL without cyclin D1 over-expression and they over-expressed cyclin D2 or cyclin D3 by gene expression profiling of cDNA.[20] As the morphology and cytogenetics of these cases had not been reported, we cannot rule out the contamination of such cases. The mechanism causing the absence of cyclin D1 expression, despite cyclin D1 and immunoglobulin gene rearrangement in this case, is currently unknown. Alternatively, the possibility of a false-negative result of CD5 and cyclin D1 should be considered; however, FL might include such cases. Further studies are required in a large series of patients.

In conclusion, the present data supported the recent idea that FL is a heterogeneous disease according to cytogenetics, histology and phenotype, although the following two points were different. First, the frequency of FL with t(14;18) in Japan was relatively high in grade 1 and transformed to aggressive large B-cell lymphoma cases, whereas it was relatively low in grade 2, 3, and FL + DLBCL cases. Second, FL with positive staining of both CD10 and Bcl-2 is more likely to have t(14;18). Molecular techniques, including FISH analysis, in daily clinical use for FL might contribute not only to an accurate diagnosis, but also novel findings regarding prognosis and therapeutic approaches.

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Diffuse Large B-Cell Lymphoma With Extra Bcl-2 Gene Signals Detected by FISH Analysis Is Associated With a "Non-Germinal Center Phenotype"

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Abstract: Amplification and translocation of the Bcl-2 gene has been detected in a certain subset of diffuse large B-cell lymphomas (DLBCL). The correlations among Bcl-2 protein expression, gene translocation or amplification, and the molecular signature determined by cDNA array are poorly understood. This study examined 25 cases with de novo nodal DLBCL. Interphase fluorescence in situ hybridization (FISH) analysis was performed to evaluate the Bcl-2 gene using IGH/BCL2 and CEP18 centromere probes (Vysis). When extra Bcl-2 gene signals were observed in each tumor cell and when these signals were in proportion to the extra CEP18 probe signals, we regarded the findings as indicating the presence of an additional chromosome 18; when extra Bcl-2 signals were observed but additional CEP18 signals were not, we regarded the findings as indicating the presence of gene amplification. A panel of 3 antigens (CD10, Bcl-6, and MUM-1) was applied to categorize each case as either a "germinal center B-cell (GCB) phenotype" or a "non-GCB phenotype." Of the 25 cases examined, 8 cases (32%) were classified as "GCB phenotype" and 17 cases (68%) were classified as "non-GCB phenotype." A FISH analysis revealed that t(14;18) was detected in 2 of the 8 cases (25%) with the "GCB phenotype" but in none of the 17 "non-GCB phenotype" cases. Extra Bcl-2 gene signals were detected in 7 of the 25 (28%) cases examined: n = 5 for an additional chromosome 18, n = 1 for gene amplification, and n = 1 for additional chromosome 18 + gene amplification. Extra Bcl-2 gene signals were exclusively detected in DLBCL with the "non-GCB phenotype"; these cases, with the exception of one, stained strongly positive for Bcl-2. The DLBCLs with Bcl-2 protein overexpression were classified into at least two heterogeneous molecular groups, based on the results of the FISH analysis.

Key Words: DLBCL, FISH, t(14;18), gene amplification

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Translocation (14;18)(q32;q21) is not specific to follicular lymphomas but also exists in 20% to 30% of patients with diffuse large B-cell lymphoma (DLBCL).[18,27] This translocation leads to the overexpression of the Bcl-2 protein by placing the Bcl-2 gene under the constitutive activation of the IgH enhancer.

The Bcl-2 protein is located in the inner membrane of the mitochondria and functions as an anti-apoptotic protein, inhibiting cells from programmed cell death.[15] Bcl-2 protein overexpression has been reported to occur in 24% to 66% of DLBCLs, a much higher frequency than that expected based on the presence of t(14;18) alone.[3,7,9,13,14,19a,22] Therefore, the presence of t(14;18) alone does not explain the mechanism responsible for the observed increase in Bcl-2 protein expression in DLBCL. The amplification of the Bcl-2 gene, as shown using comparative genomic hybridization (CGH), has been suggested to be an important alternative mechanism of Bcl-2 protein overexpression in DLBCL.[20,21,24]

As recently reported, lesions carrying t(14;18) might represent the histologic transformation of a prior follicular lymphoma or they might be associated with a DLBCL subgroup characterized by a germinal center B-cell gene expression profile.[4,16] Bcl-2 protein overexpression is also frequently present in cases without t(14;18), especially in non-GCB subgroups, which characteristically have a high level of Bcl-2 mRNA.[17] These findings suggest that the mechanism of Bcl-2 protein overexpression in non-GCB DLBCL subgroups may differ from that in the GCB subgroup.

Using immunohistochemistry to detect three antigens, the GCB and non-GCB subgroups of DLBCL can be accurately assigned to categories defined by a cDNA microarray.[11] This

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method is useful because it is widely available. However, the correlations among Bcl-2 protein expression, gene translocation or amplification, and the molecular signature determined by the cDNA array are poorly understood.

To evaluate these correlations, we examined 25 newly diagnosed nodal DLBCL specimens using interphase fluorescence in situ hybridization (FISH) and immunohistochemistry on paraffin-embedded tissues.

MATERIALS AND METHODS

Cases and Samples

This retrospective study examined 25 previously untreated cases with de novo nodal DLBCLs. All the patients had been diagnosed between June 1997 and December 2001 and had been followed up at a single institution (National Cancer Center Hospital, Tokyo, Japan). Patients with primary extranodal disease, which is more common in Japan than in other countries,[28] were excluded because these cases are rather heterogeneous. Tissue biopsies were performed before chemotherapy, and the resulting specimens were fixed in 10% buffered formalin and embedded in paraffin. All cases were reviewed by two hematopathologists (A.M., Y.M.) to confirm that the morphologic characteristics fulfilled the criteria of the REAL/WHO classification for DLBCL lymphoid neoplasms.[10,12] Cases with a history of previous low-grade B-cell lymphoma with subsequent clinical and pathologic transformation into DLBCL were also excluded.

Immunohistochemical Studies

Immunohistochemical studies were performed using a panel of monoclonal and polyclonal antibodies reactive in paraffin-embedded tissue sections according to the avidin-biotin-alkaline phosphatase complex method (Table 1). Immunoreactivity was judged positive if 20% or more of the tumor cells were stained and strongly positive if 50% or more were stained.

To classify each case as having either a "GCB phenotype" or a "non-GCB phenotype," a panel of three antigens, CD10, Bcl-6, and MUM-1, was evaluated following the protocol reported by Hans et al.[11] Briefly, cases were assigned to the "GCB phenotype" if the specimens were only positive for CD10. If the specimens were negative for both Bcl-6 and CD10, the corresponding cases were assigned to the "non-GCB phenotype." If the specimens were positive for Bcl-6 and negative for CD10, the expression of MUM1 was used to determine the group: if MUM1 was negative, the case was assigned to the "GCB phenotype"; if MUM1 was positive, the case was assigned to the "non-GCB phenotype."

TABLE 1. Antibodies Used for Immunohistochemistry

Antibody	Clone	Source*	Dilution	Antigen Retrieval
CD20	L26	Dako	1:100	Citrate 10
CD79a	JCB117	Dako	1:100	Citrate 10
CD5	4C7	Novo	1:100	Citrate 10
CD10	56C6	Novo	1:200	Citrate 10
Bcl-6	Polyclonal	Dako	1:50	TRS
MUM-1	M17	Santa Cruz	1:200	Citrate 4
Bcl-2	124	Dako	1:100	Citrate 10

Citrate 10, 10 minutes at 121°C in citrate (10 mM, pH 6.0); TRS, 10 minutes at 121°C in Target Retrieval Solution by Dako; Citrate 4, 4 minutes at 121°C in citrate (10 mM, pH 6.0).

*Dako, DakoCytomation Denmark A/S, Denmark; Novo, Novocastra Laboratories, Newcastle upon Tyme, UK; Santa Cruz, Santa Cruz Biotechnology, CA.

Interphase Fluorescence In Situ Hybridization Studies and Analysis

To perform FISH studies, 4-μm sections were cut from each paraffin block. The specimens were treated with a 2 × saline sodium citrate buffer (SSC, pH 7.3), digested with 0.005% and 0.3% pepsin/0.01 N HCl for 14 minutes at 37°C, rinsed in 1 × phosphate buffer saline (PBS, pH 7.4) for 5 minutes, formalin $MgCl_2$/PBS for 10 minutes, rinsed in 1 × PBS for 5 minutes twice, and dehydrated in an ethanol series. Next, the samples were denatured in 70% formamide/20 × SSC for 2 minutes at 73°C and dehydrated with 70% ethanol for 5 minutes, followed by 100% ethanol. Denatured probes (10 μL) were dropped onto the slides, covered with a coverslip, and sealed with rubber cement. The slides were then treated using a microwave procedure to intensify the signals. The microwave to (MI-77, Azumaya Company, Tokyo, Japan) was set to irradiate the samples for 3-second periods at intervals of 2 seconds, for a total of 60 minutes at a frequency of 2.45 GHz and an output power of 250 W with the temperature sensor set to 37°C (Kitayama, 1999[19]). After incubation overnight at 37°C, the slides were washed with 50% formamide/2 × SSC for 10 minutes at 45°C, and then washed twice more for 10 minutes each at room temperature; the slides were then washed with 2 × SSC for 10 minutes. The specimens were rinsed in 4 × SSC/0.05% Triton for 5 minutes, 2 × SSC for 5 minutes at 45°C, and 0.2 × SSC at room temperature. The slides were covered with anti-fade solution and viewed under a BX60 fluorescence microscope (Olympus, Tokyo, Japan) using a 100 × oil immersion lens and appropriate filters. This FISH assay was validated in an analysis of a series of typical follicular lymphoma cases that used the same method described above.[25]

For FISH, a dual-color LSI IgH Spectrum Green/LSI BCL2 Spectrum Orange Dual Fusion Translocation Probe (Vysis, Downers Grove, IL) was used to detect the t(14;18). If extra Bcl-2 gene signals were detected, a CEP18 Spectrum Orange probe (Vysis) was applied. To analyze the hybridization, a total of 50 to 200 nuclei per case were scored for the presence of the t(14;18) and extra Bcl-2 gene signals.

When extra Bcl-2 gene signals were observed in each tumor cell and when these signals were in proportion to the extra CEP18 probe signals, we regarded the findings as indicating the presence of an additional chromosome 18; when extra Bcl-2 gene signals were observed but additional CEP18 signals were not, we regarded the findings as indicating the presence of gene amplification (Fig. 1).

Statistical Analysis

The χ^2 test was used to analyze the relationship between variables. Overall survival (OS) was measured from the date of

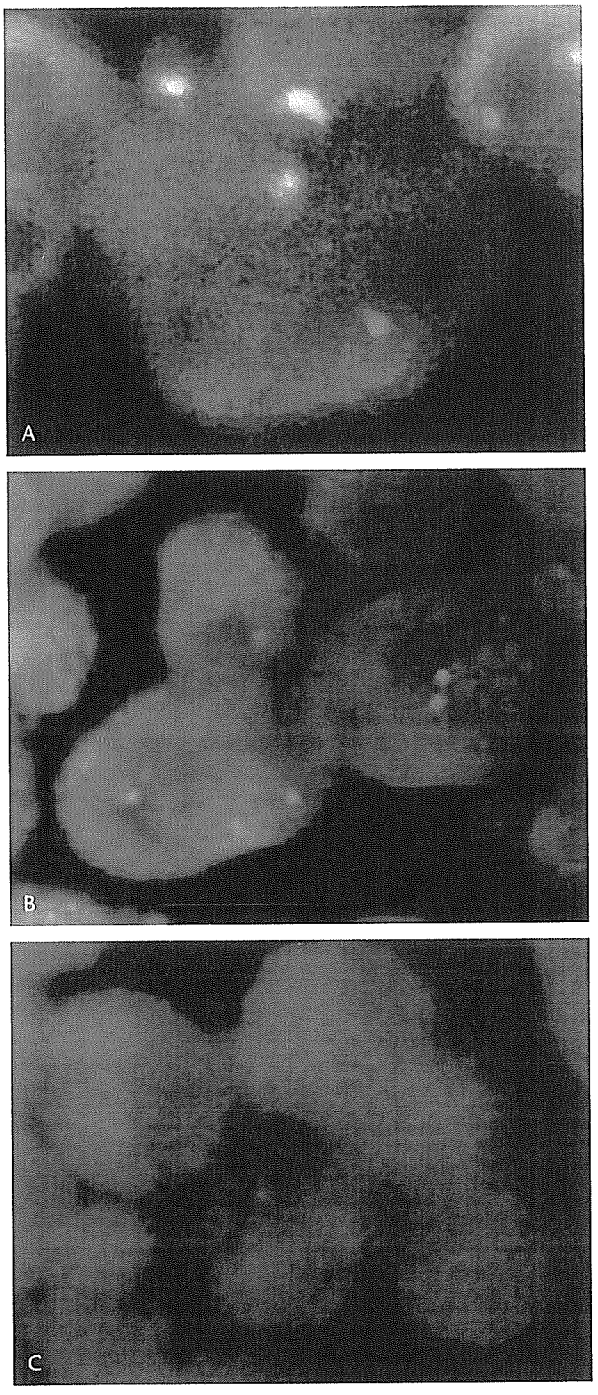

FIGURE 1. A representative FISH image. A, Presence of fusion using a green IgH signal and a red BCL2 signal. B, Multiple copies of the BCL2 signal. C, An extra chromosome 18.

diagnosis until the date of death or last contact. The survival curves were calculated using the Kaplan-Meier method, using the log-rank test to analyze the statistical differences between the groups. The statistical analysis was performed using the SPSS software package (SPSS version 11.0.1 J, SPSS Inc, Chicago, IL).

RESULTS

Clinical Characteristics and Treatment of the Cases

The clinical characteristics of the 25 cases with DLBCL are summarized in Table 2. Twelve men and 13 women with an overall median age of 60 years at the time of diagnosis (range, 24–83 years) were examined. All the DLBCLs were of primary nodal origin. Twelve percent of the cases had a poor performance status (PS $\geq$ 2), while 16% had B symptoms. The distribution according to the Ann Arbor clinical stage was as follows: I, 12%; II, 32%; III, 36%; and IV, 20%. Six cases presented with the involvement of one or more extra nodal sites, including 1 case with bone marrow infiltration. The distribution according to the International Prognostic Index (IPI) was as follows: low risk, 11 cases (44%); low-intermediate risk, 4 cases (16%); high-intermediate risk, 7 cases (28%); and high risk, 3 cases (12%).

The initial treatment, best response, current status, and OS of the 25 cases are shown in Table 2. Nearly all the cases received the standard CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) regimen at a curative intensity, with the exceptions of 1 case with a progressive disease and 1 other case with a poor PS. Ten cases (40%) received radiotherapy 36 Gy to 46 Gy after chemotherapy. Data from all 25 cases were assessed for response to initial treatment and OS. The median follow-up period for all 25 cases was 42 months (range, 3–83 months). The complete response (CR) rate was 19 of 25 patients (76%). Eighteen patients, including the 13 who had been in continuous first CR, were alive at the time of the last follow up.

Immunohistochemical Data

The results of the immunohistochemical staining for each antigen are shown in Table 3. All the specimens were CD20- and CD79a-positive, but none of the specimens was CD5-positive in this study. CD10, Bcl-6, and MUM-1 were positive in 5 of 25 (20%), 24 of 25 (96%), and 17 of 25 (68%) specimens, respectively. Nineteen of the 25 cases (76%) were positive and 13 (52%) were strongly positive for Bcl-2 protein staining.

Following the protocol reported by Hans et al,[11] 8 cases (32%) were classified as "GCB phenotype" and 17 cases (68%) were classified as "non-GCB phenotype" (Table 3).

The overexpression of Bcl-2 protein was observed in 5 of the 8 cases (63%) with the "GCB phenotype" and in 14 of the 17 cases (82%) with the "non-GCB phenotype."

Detection of t(14;18) and Extra Bcl-2 Gene Signals by FISH Analysis

FISH analysis revealed that t(14;18) was detected in 2 of the 8 cases (25%) with the "GCB phenotype" but in none of the 17 cases with the "non-GCB phenotype." Extra Bcl-2 gene signals were detected in 7 of the 25 cases (28%): 5 with an additional chromosome 18, 1 with gene amplification, and 1 with an additional chromosome 18 plus gene amplification (Tables 3, 4).

Only DLBCL specimens with a "non-GCB phenotype" exhibited findings characteristic of an additional chromosome

TABLE 2. Clinical Characteristics and Clinical Courses of 25 Cases of De Novo Nodal Diffuse Large B-Cell Lymphoma

Case No.	Age (yr)/Sex	PS	CS	IPI	Initial Treatment	Best Response	Current Status	Overall Survival (day)
1	62/F	1	IIIA	High-intermediate	CHOP × 7	CR	CR1	1789+
2	60/M	1	IIIA	Low-intermediate	CHOP × 8, RT	CR	CR2	1258+
3	71/F	3	IVB	High	CHOP × 8	PR	Dead	200
4	60/M	1	IIA	Low	CHOP × 8, RT	CR	CR1	1919+
5	60/F	0	IA	Low	CHOP × 3, RT	CR	CR1	1244+
6	67/F	0	IIIA	High-intermediate	CHOP × 8	CR	Dead	678
7	63/M	0	IIIA	High-intermediate	CHOP × 8	PR	CR1	1593+
8	53/F	0	IIA	Low	CHOP × 7	CR	CR1	1464+
9	27/F	0	IIIA	Low-intermediate	CHOP × 8	CR	CR1	2306+
10	83/M	2	IIA	High-intermediate	CHOP × 8, RT	CR	CR1	2436+
11	66/M	3	IVB	High	CHOP × 8	CR	PR2	2005+
12	73/F	1	IVA	High	CHOP × 10	CR	CR1	1272+
13	54/M	0	IIB	Low	CHOP × 6, RT	CR	CR1	953+
14	63/M	0	IIA	Low-intermediate	CHOP × 8, RT	CR	CR2	1289+
15	34/M	1	IA	Low	CHOP × 1	PD	Dead	367
16	35/M	0	IIIA	Low	CHOP × 8	CR	Dead	699
17	81/F	0	IIIA	High-intermediate	Oral etoposide	NR	Dead	93
18	65/M	0	IIA	Low	CHOP × 6, RT	CR	CR1	2034+
19	41/F	0	IIA	Low	CHOP × 8, RT	CR	CR1	1671+
20	59/F	0	IA	Low	CHOP × 3, RT	CR	CR1	1036+
21	63/M	0	IIIA	High-intermediate	CHOP × 8	PR	Dead	963
22	40/F	0	IIIA	Low-intermediate	CHOP × 8	CR	CR1	927+
23	60/F	0	IIA	Low	CHOP × 8, RT	CR	CR2	2494+
24	24/M	1	IVB	High-intermediate	CHOP × 8	PR	Dead	302
25	58/F	1	IVA	Low	CHOP × 8	CR	CR2	931+

PS, performance status by the Eastern Cooperative Oncology Group scale; LDH, lactate dehydrogenase; CS, clinical stage; CHOP, cyclophosphamide, adriamycin, vincristine, prednisolone; CR, complete response; PR, partial response; NR, no response; IPI, international prognostic index; RT, radiation therapy; PD, progressive disease.

18 and gene amplification, and these specimens, except for one, were strongly positive for Bcl-2.

The 13 cases that were strongly positive for Bcl-2 included two with t(14;18) and six with extra Bcl-2 gene signals. None of the specimens exhibited both t(14;18) and extra Bcl-2 gene signals simultaneously. Furthermore, 5 cases that were strongly positive for Bcl-2 staining did not exhibit either the translocation or extra Bcl-2 gene signals.

Correlation Between FISH Alterations and Clinical Characteristics and Outcome

No significant correlations between the presence of the extra Bcl-2 gene signals and age, advanced stage, high serum lactate dehydrogenase levels, poor PS, high IPI score, CR rate, or OS were observed ($P > 0.05$).

DISCUSSION

In the present study, CD10, Bcl-6, and MUM-1 were immunohistochemically used to categorize the specimens into either a "GCB phenotype" or a "non-GCB phenotype." Several protocols for the immunohistochemical characterization of "GCB" and "non-GCB phenotypes" have been reported. The immunohistochemical data obtained in the present study, using the protocol reported by Hans et al,[11] were well correlated with the cDNA array data. The present immunohistochemical protocol uses only three antigens to define the GCB phenotype and the non-GCB phenotype.[11] The phenotypes assigned using this method and those defined using the cDNA array had the same prognostic ability. This method was also used by Chang et al, who reported that tissue microarrays can be used to predict patient prognosis with the same accuracy as that of cDNA arrays.[6]

Using this protocol, we found that 8 of the 25 specimens (32%) examined in this study exhibited a "GCB phenotype" and that 17 (68%) exhibited a "non-GCB phenotype." The percentage of DLBCL specimens with a "GCB phenotype" was significantly lower than those percentages reported in previous papers by Rosenwald et al[24a] (48%) and Alizadeh et al[1] (50%). The percentage in the present study was also lower than that determined using a tissue array (42%).[11] We omitted DLBCLs of extranodal origin, among which the "non-GCB phenotype" is relatively common[9]; if these extranodal cases had been included in the present study, the differences in the percentage of DLBCLs with the "GCB phenotype" between this study and the previous ones would have been even larger. These differences might be partly explained by geographic variations in the prevalence of nodal cases. In Japan, follicular lymphoma, another type of lymphoma with a germinal center origin, is relatively less common than in the United States and Europe,[1a] and a similar difference in regional prevalence might account for the paucity of DLBCL

TABLE 3. Immunohistochemistry and Fluorescence in Situ Hybridization Analysis

Case No.	CD20	CD79a	CD5	CD10	Bcl-6	MUM-1	GCB/Non-GCB	Bcl-2	FISH
1	+	+	−	+	+	−	GCB	++	t(14;18)
2	+	+	−	+	+	−	GCB	++	t(14;18)
3	+	+	−	+	+	−	GCB	++	(−)
4	+	+	−	+	+	−	GCB	++	(−)
5	+	+	−	−	+	−	GCB	+	(−)
6	+	+	−	−	+	−	GCB	−	(−)
7	+	+	−	+	+	−	GCB	−	(−)
8	+	+	−	−	+	−	GCB	−	(−)
9	+	+	−	−	+	+	Non-GCB	++	Additional chromosome 18
10	+	+	−	−	+	+	Non-GCB	++	Additional chromosome 18
11	+	+	−	−	+	+	Non-GCB	++	Additional chromosome 18
12	+	+	−	−	+	+	Non-GCB	++	Additional chromosome 18
13	+	+	−	−	+	+	Non-GCB	+	Additional chromosome 18
14	+	+	−	−	+	+	Non-GCB	++	Gene amplification
15	+	+	−	−	+	+	Non-GCB	++	Additional chromosome 18 + gene amplification
16	+	+	−	−	+	+	Non-GCB	+	(−)
17	+	+	−	−	+	+	Non-GCB	++	(−)
18	+	+	−	−	+	+	Non-GCB	+	(−)
19	+	+	−	−	+	+	Non-GCB	++	(−)
20	+	+	−	−	+	+	Non-GCB	+	(−)
21	+	+	−	−	+	+	Non-GCB	++	(−)
22	+	+	−	−	+	+	Non-GCB	+	(−)
23	+	+	−	−	−	+	Non-GCB	−	(−)
24	+	+	−	−	+	+	Non-GCB	−	(−)
25	+	+	−	−	+	+	Non-GCB	−	(−)

GCB, germinal center B-cell phenotype; FISH, fluorescence in situ hybridization; (−), no abnormality shown.

with a "GCB phenotype" in the present study. Alternatively, the difference could be the result of a sampling bias; this matter will require clarification in the future.

Our FISH analysis detected t(14;18) in 2 of the 25 specimens (8%) that were examined; both of these specimens were classified as having a "GCB phenotype." This significant finding is consistent with recent reports hypothesizing that t(14;18) might be associated with DLBCL subgroups with a GCB gene expression profile but not with activated B-cell like (ABC) subgroups,[4,7,17] although the lower percentage could also be explained by the lower percentage of the "GCB phenotype."

The use of FISH probes enables gene copy numbers to be assigned simultaneously. We observed extra Bcl-2 gene

TABLE 4. Extra BCL-2 Gene Signals by Fluorescence in Situ Hybridization Analysis

Case No.	Probe/Signal	0	1	2	3	4	5<	Total	Extra BCL-2 Gene Signals
9	BCL-2	0	22	60	17	3	0	102	Additional chromosome 18
	CEP18	0	21	66	13	2	0	101	
10	BCL-2	2	12	23	13	3	0	53	Additional chromosome 18
	CEP18	17	41	97	45	0	0	200	
11	BCL-2	3	14	20	13	2	0	52	Additional chromosome 18
	CEP18	9	30	88	61	11	0	199	
12	BCL-2	1	9	23	13	0	0	46	Additional chromosome 18
	CEP18	11	34	104	46	5	0	200	
13	BCL-2	0	2	9	23	8	11	53	Additional chromosome 18
	CEP18	0	6	28	34	20	12	100	
14	BCL-2	1	3	11	15	12	13	55	Gene amplification
	CEP18	38	70	90	3	0	0	201	
15	BCL-2	0	3	9	30	18	12	72	Additional chromosome 18 + gene amplification
	CEP18	0	6	58	27	9	0	100	

signals in 7 of the 25 cases (28%) examined. The extra signals were attributed to the presence of trisomy 18 or further additional chromosomes (5 cases), Bcl-2 gene amplification (1 case), or both (1 case). Our data were consistent with those reported by Barrans et al, who demonstrated the presence of extra Bcl-2 gene signals in 20% (27 of 137) of their DLBCL cases and attributed the extra signals to the presence of trisomy 18 (3 cases), more than three copies of chromosome 18 (17 cases), or gene amplification (7 cases).[4]

Amplification of the Bcl-2 gene, as well as chromosomal amplification involving 18q21, has also been detected in DLBCL using CGH analysis,[5,21,24] and the resulting percentage of specimens exhibiting amplification was comparable to that detected by FISH. However, the correlation between the amplification of the Bcl-2 gene and the immunohistochemical expression patterns ("GCB" or "non-GCB phenotype") observed in DLBCLs remained unclear. Therefore, we assigned each specimen to either the "GCB" or the "non-GCB phenotype" and, for the first time, showed that the specimens with extra Bcl-2 gene signals were correlated with the "non-GCB phenotype." This observation is another example of genetic change at the DNA level that correlates well with the expression profiling. A similar result was recently shown by Farinha et al,[25] who showed that 15 of 23 cases with a high Bcl-2 gene copy number exhibited a "non-GCB phenotype." It should be noted that all the specimens in the present study were allocated to the "non-GCB phenotype" after immunostaining for MUM-1; these specimens were CD10-negative and Bcl-6-positive and might have been assigned to the GCB phenotype if MUM-1 staining had not been performed.

None of the specimens simultaneously exhibited both t(14;18) and extra Bcl-2 gene signals in the present study. Since this result was consistent with the findings of other studies,[24,8,21] these mutually exclusive abnormalities might be primary events in the molecular pathogenesis of DLBCL.

In addition to the high expression level of Bcl-2 protein in the specimens carrying t(14;18), we showed that 6 of 7 specimens with extra Bcl-2 signals also had elevated levels of Bcl-2 expression, as detected by immunohistochemistry. This result was confirmed by the CGH data, which showed that the amplification of the Bcl-2 gene was a consistently important prerequisite for Bcl-2 protein overexpression in DLBCL.[21,24] This phenomenon is known as gene dosage theory.[2] However, the increased level of Bcl-2 gene expression observed in DLBCLs cannot be fully explained by only these two genetic changes. We showed that 5 specimens (20%) with strong Bcl-2 staining exhibited neither t(14;18) or additional copies of the Bcl-2 gene; 2 of these cases had a "GCB phenotype" and 3 had a "non-GCB phenotype." Farinha et al also found increased Bcl-2 gene copy numbers in 24% (23 of 94) of their DLBCL specimens,[8] in which the expression of the Bcl-2 protein was highly correlated with this FISH-detected alteration; however, another 24% (22 of 94) of their specimens were strongly positive for Bcl-2 staining but exhibited neither of the above-mentioned genetic changes, as detected by FISH. Thus, another mechanism must account for some of the DLBCLs with high Bcl-2 protein expression.

Patients with DLBCLs exhibiting a "GCB phenotype" have been reported to have a better therapeutic outcome than patients with DLBCLs exhibiting a "non-GCB phenotype."[6,11] We compared the CR rate and the OS between patients with DLBCLs exhibiting the "GCB phenotype" or the "non-GCB phenotype," but no significant differences were found (data not shown), probably because of the limited number of cases in this study. More cases are needed to validate the molecular classification of DLBCL using immunohistochemistry.

We did not observe any difference in the outcome of patients with Bcl-2-positive or Bcl-2-negative DLBCLs. The prognostic significance of Bcl-2 protein expression is controversial.[7,9,13,14,23,26] Some studies have shown that Bcl-2 protein overexpression was associated with a significantly worse OS,[7,9,13,14] but other studies have shown no differences in OS.[27,28] Recently, the overexpression of Bcl-2 protein in DLBCL was shown to be an adverse predictor only in "non-GCB subgroups" and not in "GCB subgroups."[4,11] The main purpose of the present study was to analyze these two categories separately, but the small number of cases with a "non-GCB phenotype" (n = 17) prevented this issue from being resolved.

No correlations between the FISH-detected alterations and the pathologic/clinical characteristics (centroblastic/immunoblastic, IPI score) were found, and the specimens with extra Bcl-2 gene signals did not constitute specific entities that could be attributed to the small number of cases in this cohort. Using CGH, Bea et al recently reported that DLBCLs with an 18q gain were significantly associated with primary nodal presentation, high serum lactate dehydrogenase levels, a high IPI score, shorter cause-specific survival, and a high risk of relapse.[5] Because FISH analysis is a more convenient method for identifying such cases, the analysis of a larger series to identify the characteristics of de novo DLBCL specimens with an 18q gain is desired.

In summary, our results confirmed the findings of previous reports indicating that t(14;18) is found in DLBCL specimens exhibiting a "GCB phenotype." Furthermore, an additional chromosome 18 and/or Bcl-2 gene amplification were associated with a "non-GCB phenotype." DLBCL specimens with Bcl-2 protein overexpression can be divided into at least two heterogeneous molecular groups, and this observation should be considered when analyzing the clinicopathologic features of DLBCL.

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