

***BCL6* gene amplification/3q27 gain is associated with unique clinicopathological characteristics among follicular lymphoma without *BCL2* gene translocation**

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Although approximately 10–20% cases of follicular lymphoma lack *BCL2* gene rearrangement, there are few reports having described the alternative genetic aberrations and their association about clinicopathological features. In this study, analysis by Fluorescence *in situ* hybridization of *BCL6* gene aberrations in 100 follicular lymphoma cases without *IGH/BCL2* rearrangement resulted in the identification of four subgroups. Group I: *BCL6* gene rearrangement ($n = 41$); Group II: *BCL6* gene amplification/3q27 gain ($n = 30$); Group III: the absence of both ($n = 23$); and Group IV: the presence of both ($n = 6$). Group II showed higher grade morphology (Grade 3a/b: 93%), higher *bcl2* and MUM1 expression (73 and 57%, respectively), and more frequent combination with *BCL2* gene amplification/18q21 gain (90%) than the other groups. *BCL6* gene aberration, especially amplification/3q27 gain, indicates the presence of certain morphological and phenotypical findings in follicular lymphoma cases without *IGH/BCL2* rearrangement.

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Follicular lymphoma is the most prevalent form of low-grade B-cell lymphoma in adults.¹ t(14;18)(q32;q21), the *BCL2* oncogene rearrangement accompanied by constitutive expression of the *bcl-2* protein, characterizes most cases of follicular lymphoma and is a critical event in lymphomagenesis.² However, approximately 10–20% cases of follicular lymphoma lack *BCL2* rearrangement.^{1,3} Recent reports have described some alternative genetic aberrations for t(14;18)(q32;q21)-negative follicular lymphoma, for example, *BCL6* gene rearrangement^{3–8} and trisomy 18.⁹ We previously performed a high-resolution bacterial artificial chromosome

(BAC) array CGH for 24 t(14;18)-negative follicular lymphoma cases.¹⁰ We detected trisomy 3, which is genomically characterized by gain/amplification of 18/18q, in part of t(14;18)-negative follicular lymphoma, and this aberration was mutually exclusive with *BCL6* rearrangement. Furthermore, CD10(–)MUM1(+) phenotype, which suggests that this subtype may be derived from late germinal center B cells,^{10,11} was frequently observed in follicular lymphoma with trisomy 3. These findings suggest that follicular lymphoma with *BCL6* rearrangement and follicular lymphoma with trisomy 3 (including *BCL6* gene) form a unique subgroup in follicular lymphoma without *BCL2/IGH* rearrangement. But there has been no clinicopathological study to compare these subgroups of follicular lymphoma without *BCL2/IGH* rearrangement.

In this study, we divided follicular lymphoma without *BCL2/IGH* rearrangement into four genetical subtypes based on *BCL6* gene aberration status and compared their clinicopathological features.

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Materials and methods

Biological Material

Tissue specimens were obtained from human samples filed at the Department of Pathology of Kurume University between 2000 and 2007. Paraffin-embedded tissues were available in all cases. Cell suspensions were used for fluorescent *in situ* hybridization. These studies were agreed to by the patients or their legal guardians, whereas appropriate written consent was obtained from each participating institute or hospital.

Morphological Criteria

Follicular lymphoma was diagnosed according to new WHO classification with at least a partially follicular pattern.¹ Three experienced pathologist (KK, GY, and OK) reviewed all cases. If the case had more than 50% of diffuse area, we defined these cases as follicular lymphoma with diffuse area. In some cases, (1) follicular structures are not definitive; (2) lymphoma cells showed abundant clear cytoplasm and slightly indented nuclei, namely 'marginal zone differentiation;' and (3) lymphoma cells showed predominant marginal zone invasion surrounding lymph follicles rather than follicular invasion. We deleted such cases from study because it is difficult to differentiate them from nodal marginal zone lymphoma.

Immunohistochemistry

Paraffin sections from each sample were immunostained with monoclonal antibodies against CD20 (DAKO, Glostrup, Denmark), CD3 (Novocastra, Newcastle, UK), CD10 (Novocastra), Bcl2 (DAKO), MUM1 (DAKO), and Bcl6 (Novocastra) with a previously reported method.¹² Two categories were defined as negative (<30% positively stained tumor cells) and positive (\geq 30% positively stained tumor cells).

Cytogenetical Analysis and Fluorescence *In Situ* Hybridization

The usual G-banding method was used for cytogenetic analysis. Karyotypes were described according to the International System for Human Cytogenetics Nomenclature (1995).

Cell suspensions fixed with methanol/acetic acid (3:1) were hybridized with the LSI IgH Spectrum Green/LSI Bcl2 Spectrum Orange Dual-Fusion Translocation Probe (Vysis, Downers Grove, IL, USA) and the LSI Bcl6 Dual Color Breakpoint Probe (Vysis). Analyses were performed with previously described methods.¹³

Briefly, the fluorescence *in situ* hybridization (FISH) patterns were interpreted after counting 200

nuclei. A specimen was classified as abnormal and consistent with *IGH/BCL2* fusion, *BCL6* break, extra copies of *BCL2* gene, or extra copies of *BCL6* gene, when the number of nuclei observed with these abnormal patterns was >5%.

Statistical Analysis

Student *t*-test and χ^2 test were used to compare clinical and pathological findings among the four groups. A *P*-value of less than 0.05 represents a statistically significant difference.

Results

Clinicopathological Features of Follicular Lymphoma without *BCL2* Rearrangement

Of all 609 follicular lymphoma cases enrolled, 519 were analyzed with G-banding and FISH using the *BCL2/IgH* probe. Sixteen cases, already analyzed in previous study, were included¹¹ (Table 1). The presence of t(14;18)(q32;q21) detected by G-banding or *IGH/BCL2* with FISH rearrangement resulted in the exclusion of 387 cases as follicular lymphoma with *BCL2* gene rearrangement. Of the 132 follicular lymphoma cases without *BCL2* rearrangement as evidenced by not only G-banding but also FISH, 100 cases with sufficient materials were suitable for further analysis. All 100 cases were confirmed as the primary diagnostic biopsies. No secondary biopsies or recurrences after therapy were included. Fifty-nine cases were successfully analyzed by G-banding method (Supplementary File 1). Additional analysis of all cases of follicular lymphoma without *BCL2* rearrangement for *BCL6* gene aberrations by FISH showed that 41 cases featured only *BCL6* rearrangement (Figure 2a) and 30 cases only *BCL6* amplification/3q27 gain (Figure 2b). Six cases possessed both *BCL6* abnormalities and 23 did not possess either aberration. This indicates that the simultaneous occurrence of *BCL6* rearrangement and *BCL6* amplification/3q27 gain is relatively rare. Of the 36 cases with *BCL6* amplification/3q27 gain, 33 possessed three or four signals in one lymphoma cell. Although three cases had more than four signals, two of them, which were analyzed by G-banding also, showed obvious amplification of chromosomes, including chromosome 3 (case nos. 27 and 29, Supplementary File 1). Although we could not confirm the chromosomal duplication by using centromeric probes, these findings indicate that *BCL6* amplification/3q27 gain reflects chromosome 3 duplication, including chromosome 3 trisomy, rather than tandem *BCL6* gene amplification. As for *BCL6*, 32 of the 36 cases with *BCL2* amplification/18q21 gain were found to possess chromosome 18 duplication, and not tandem *BCL2* gene amplification (data not shown).

Table 1 Clinicopathological features of follicular lymphoma without *BCL2*/*IgH* rearrangement

	Grade 3a/b (%)	<i>BCL2</i> amplification/ trisomy 18 (%)	<i>CD10</i> (%)	<i>MUM1</i> (%)	<i>Bcl2</i> (%)	<i>Bcl6</i> (%)	Diffuse area (%)	Age(median) (years)	Male (%)	<i>B</i> symptom (%)	<i>LDH</i> > <i>NR</i> (%)	<i>PS</i> > <i>1</i> (%)	Stage III/IV (%)
Group I (<i>n</i> =41)	41] 1	7.30] 3	46	25] 5	29	75	41	45–85 (64)	44	22	20	58	58
Group II (<i>n</i> =30)	93] 4	90] 4	33	57] 6	73 } 7	67	47	23–89 (63)	66	33	17	38	71
Group III (<i>n</i> =23)	39] 2	8.60	70	0	26 } 8	87	26	33–86 (67)	74	5	6	27	29*
Group IV (<i>n</i> =6)	83	67	83	17	33	67	33	53–76 (64)	50	50	33	33	50

NR, normal range; PS, performance status.

Group I: *BCL6* gene translocation, Group II: *BCL6* gene amplification/3q27 gain, Group III: absence of both, and Group IV: presence of both.

1–8: Significantly different from Groups I and III (*P*-value: 1: <0.00001, 2: 0.00002, 3: <0.00001, 4: <0.00001, 5: 0.006, 6: 0.000012, 7: 0.00024, and 8: 0.00064).

*Significantly lower than Group II (*P*-value = 0.02).

Table 1 shows the clinicopathological features of follicular lymphoma without *BCL2*/*IgH* rearrangement. Morphologically, 60 cases (60%) showed Grade 3a/b (3a: 33% (33 cases), 3b: 27% (27 cases)), whereas immunohistochemically, 56% were found to be positive for *CD10* and 42% for *bcl2*.

Genetic Subgroup of Follicular Lymphoma without *BCL2* Rearrangement

We divided follicular lymphoma without *BCL2* rearrangement into four subgroups: Group I: *BCL6* gene rearrangement/3q27 translocation (*n*=41); Group II: *BCL6* gene amplification/3q27 gain (*n*=30); Group III: the absence of both (*n*=23); and Group IV: the presence of both (*n*=6). Most of the cases were assigned to Groups I, II, and III, and relatively few to Group IV. *BCL2* amplification/18q21 gain was detected in no less than 90% of Group II, but only 7.3 and 8.6%, respectively, each of Groups I and III possessed this aberration (*P*<0.001). This finding proves the strong relationship between *BCL6* amplification/3q27 gain and *BCL2* amplification/18q21 gain. We summarized the cytogenetic abnormalities focusing on chromosomal abnormalities associated with gain in 59 cases with G-band data to rule out the possibility that this relationship was the result from the widespread tendency for chromosomal gain as an underlying mechanism (Supplementary File 1). To identify the specific abnormalities and avoid the secondary changes, the stemline karyotype (the simplest abnormal clone) was used as the representative karyotype for all analyses. In patients who had composite karyotypes or when stemline karyotypes could not be specified, we used the karyotype that were most encountered. As shown in Table 2, Group II had more abnormalities associated with gain compared with Groups I and III (average: Group II: 4, Group I: 1.7, and Group III: 2.1). Furthermore, the amplification associated with 3q27 and 18q32 were preferential (21/52; 40%) rather than only a part of widespread tendency of chromosomal gain in Group II.

Morphologically, 59% of Group I and 61% of Group III were low grade (Grade 1 or 2), but most of Group II (93%) was high grade (Grade 3a/b) (*P*<0.001) (Figures 1 and 2). Fourteen cases (47%) of Group II were Grade 3b and this proportion was significantly higher than Group I (10 cases, 25%) and Group III (0 cases) (*P*<0.05). Immunohistochemistry showed higher *bcl2* and *MUM1* expression in Group II (73 and 57%) than in Group I (29 and 25%) or Group III (26% and 0%) (*P*<0.001). Follicular lymphoma with *CD10*-*MUM1*+ phenotype is included in follicular lymphoma without *BCL2* rearrangement and accounted for 15 cases in Group II (50%), which is significantly higher than in Groups I and III (15 and 0%, respectively; *P*<0.005). Group III showed similar pathological characteris-

Table 2 Chromosomal changes in each group

Group and number of cases analyzed by G-band	Cases with any chromosomal gain	Total chromosomal gain events among positive cases (average per case)	Gain including 3q27 and 18q32 abnormalities among total chromosomal gain events
Group I (n = 26)	9 (35%)	15 (1.7)	0 (0%)
Group II (n = 15)	13 (87%)	52 (4)	21 (40%)
Group III (n = 14)	9 (64%)	19 (2.1)	3 (16%)
Group IV (n = 4)	2 (50%)	11 (5.5)	4 (36%)
	1: $P=0.0012$, 2: $P=0.16$, χ^2 test	3: $P=0.003$, 4: $P=0.02$, Student <i>t</i> -test	5: $P=0.003$, 6: $P=0.052$, χ^2 test

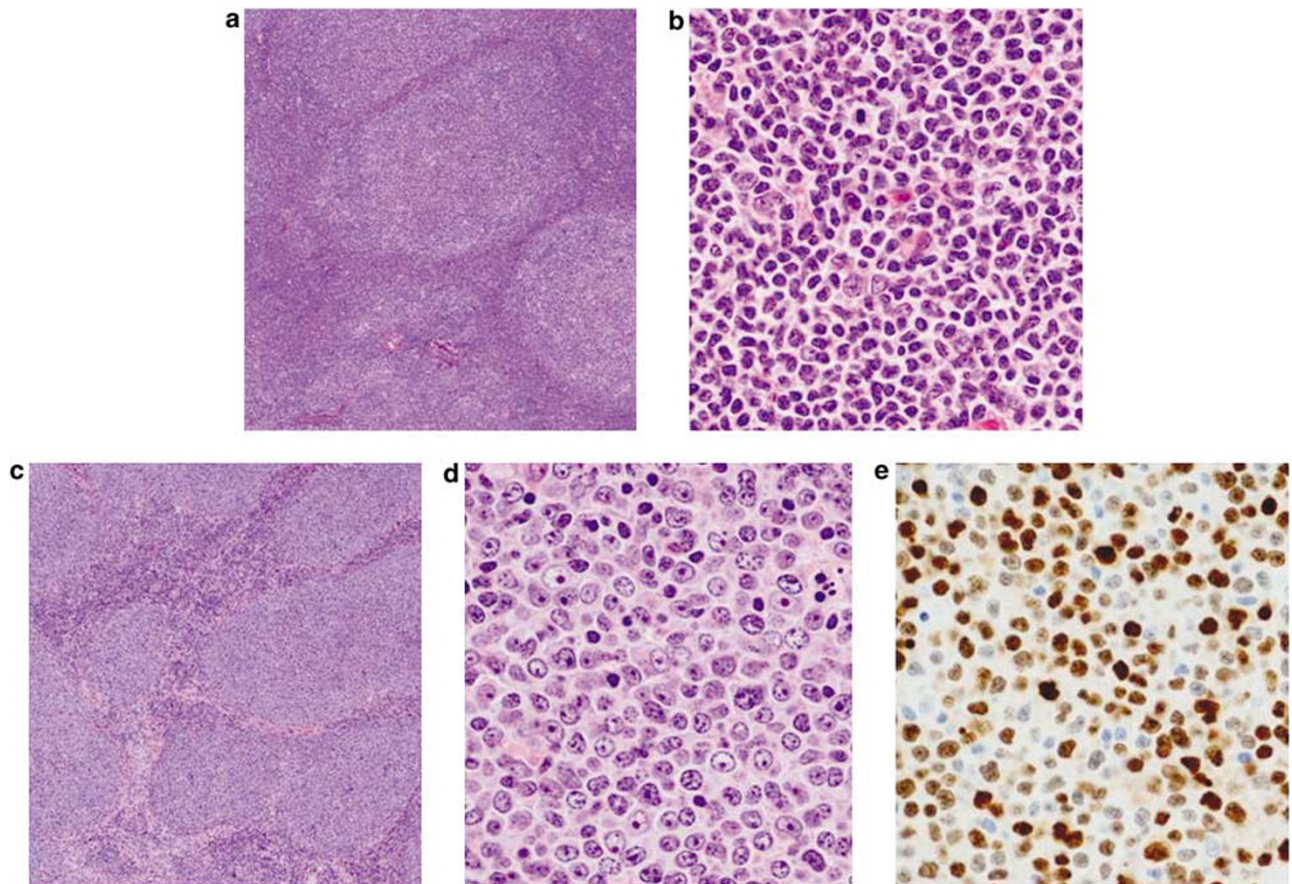


Figure 1 Morphological features of follicular lymphomas without *BCL2* gene translocation. (a, b) From a representative case of Group I and (c–e) from a case categorized into Group III. (a, c) In H&E sections, the lymphoma cells form definite follicular structures. As shown in (b), more than half of Group I cases were diagnosed as low grade (Grade 1 or 2), whereas almost all of the Group III cases were diagnosed as high grade (Grade 3a or 3b) (c). (e) More than half of Group III cases were positive for MUM1. Original magnification: (a, c): $\times 25$, (b, d, e): $\times 200$.

tics to those of Group I. The ratios of high-grade morphology (39%), *BCL2* amplification/18q21 gain (8.6%), CD10 positivity (70%), *bcl2* positivity (26%), and *bcl6* positivity (87%) were not significantly different from those in Group I, but MUM1 positivity was different (MUM1: 0 vs 25%, $P < 0.01$). Group IV had only a small number of cases ($n = 6$), indicating *BCL6* gene rearrangement and amplification/3q27 gain was uncommon. Therefore, definite comparisons with other groups were difficult.

Clinical information about male/female ratio and age was collected for all cases, and other clinical findings (B symptoms (89 cases), LDH level (79 cases), performance status (46 cases), and clinical stage (57 cases)) were partly obtained.

There were generally no significant differences between clinical features among groups. However, Group III showed relatively favorable clinical features as well as infrequent occurrence of LDH elevation (5%) and B symptom (5%) compared with

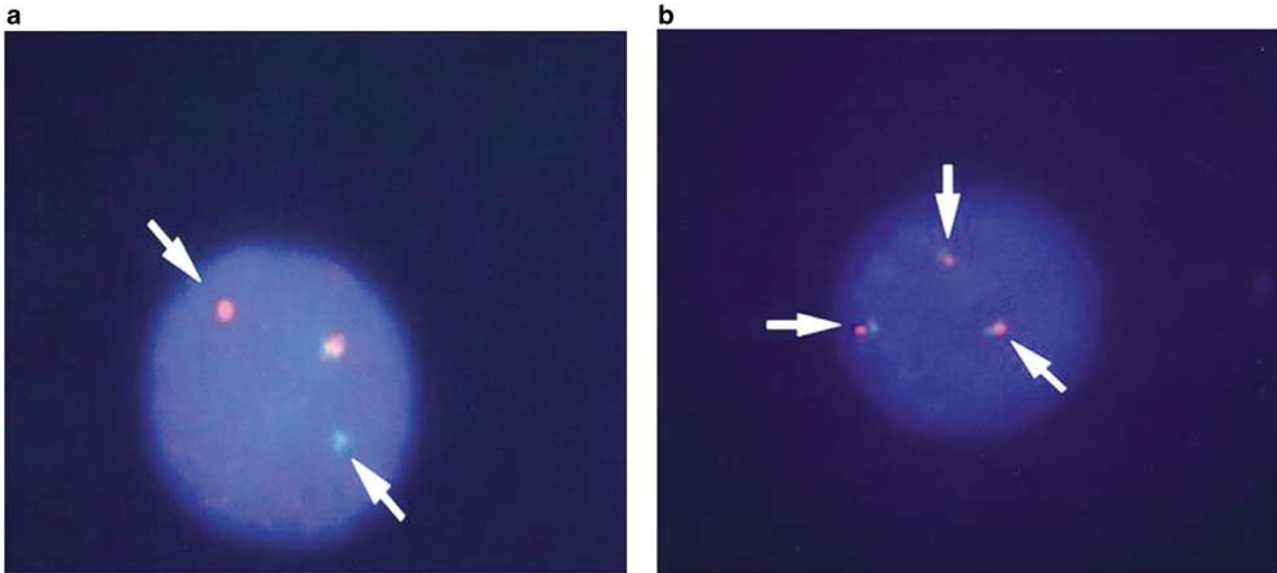


Figure 2 Representative result of dual-color FISH analysis for *BCL6* (3q27). Rearrangement (a) and extra copies (b) of *BCL6* (3q27) are detected.

other major groups (LDH, Groups I and II: 20 and 17%; B symptom, Groups I and II: 22% ($P=0.08$) and 33% ($P=0.02$)). Group III contained relatively few advanced stage cases (Stage III/IV: 29%) compared with other groups (Groups I and II: 58 and 71%). Only Groups II and III showed a significant difference ($P=0.02$).

Discussion

In this study, we performed morphological, immunohistochemical, and genetical review of follicular lymphoma without *BCL2* rearrangement. Follicular lymphoma without *BCL2* rearrangement showed high-grade morphology and relatively low CD10 and *bcl2* expression compared with follicular lymphoma with *BCL2* rearrangement. These findings are compatible with those of previous reports including our study.^{3,4,9–11,14} In such cases, marginal zone lymphoma with follicular colonization and marginal zone lymphoma with vague nodular pattern were considered as differential diagnosis. But we confirmed they are follicular lymphoma because (1) all cases were lymph node lesion, not extranodal sites, (2) all of them showed distinct follicular growth pattern, (3) and lymphoma cells are composed of medium-sized cells with angulated, elongated, twisted, or cleaved nuclei and scant pale cytoplasm known as ‘centrocytes,’ and large transformed cells with round or oval nuclei, vesicular chromatin, and a narrow rim of cytoplasm known as ‘centroblasts,’ which are morphological hallmark of follicular lymphoma.

In follicular lymphoma with *BCL2* rearrangement, the *BCL2* oncogene is relocated into an immunoglobulin gene locus, leading to deregulated expression

of the *bcl2* protein. This rearrangement causes abrogation of apoptosis, and it is considered to be the initial etiological mutation.^{2,15,16} In contrast, it has not been determined which genetic aberration is the etiological trigger of lymphomagenesis in follicular lymphoma without *BCL2* rearrangement. *BCL6* gene translocation is a representative gene aberration in follicular lymphoma without *BCL2* rearrangement.^{3,4,17} Previous studies have reported that follicular lymphoma with *BCL6* rearrangement is frequently observed in high-grade (Grade 3) follicular lymphoma cases.^{3,7} In our study, however, more than half of the follicular lymphoma cases with *BCL6* rearrangement (59%) showed low-grade morphology, whereas its morphological and phenotypical features, especially in the *bcl6* protein, were not significantly different from those of follicular lymphoma cases without any *BCL6* gene aberration (Group III). These findings indicate that the rearrangement of the *BCL6* gene in follicular lymphoma may not perform as important a role, especially in transformation, as previously reported. Interestingly, a recent study on IgH S region break points in t(3;14)(q27;q32) (*BCL6/IgH*) demonstrated that in follicular lymphoma most IgH break points involved S_{γ} , whereas in diffuse large B-cell lymphoma mostly S_{μ} -regions are involved. The S_{γ} -break point in follicular lymphoma implies that *bcl6* expression is driven by an I_{γ} -promoter whose activation requires CD40 and cytokine signaling, whereas the S_{μ} -break point leads to *BCL6* expression driven by the constitutively active intronic IgH enhancer.¹⁸ Another study found that the *BCL6* gene rearrangement did not affect *bcl6* mRNA expression.¹⁹ The significance of *BCL6* gene rearrangement in follicular lymphoma, therefore, deserves to be discussed again on the basis of further evidence.

In contrast, follicular lymphoma with *BCL6* amplification/3q27 gain (Group II) showed significant pathological characteristics (high-grade morphology, *BCL2* gene amplification/18q21 gain and *bcl2* expression and CD10-MUM1+ phenotype¹¹). In this group, *BCL2* gene amplification/18q21 gain was thought to be due to trisomy 18, which possibly implicate the effect of an increased dosage, and related to lymphomagenesis similar to that seen in follicular lymphoma with *BCL2* rearrangement as previously reported.⁹ Almost all cases in which G-banding analysis was successfully performed (15 cases) showed complex chromosomal aberrations, not only trisomy 3 and 18 (Supplementary File 1), and these numerous aberrations may be involved in the transformation/high-grade morphology.

In conclusion, follicular lymphoma without *BCL2* gene translocation possesses heterogenous biological characteristics, two of which are *BCL6* gene amplification/3q27 gain and *BCL6* gene translocation. The former is associated with definitive clinicopathological features, whereas the latter has relatively less effect on pathological characteristics. In this study, we could not follow up the clinical prognosis of these cases, but it can be expected that analysis of prognosis will disclose more significant clinical differences among these groups.

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References

- 1 Nathwani BN, Harris NL, Berger F, *et al.* Follicular lymphoma. In: Jaffe ES, Stein H, Vardiman JW (eds). Pathology and Genetics of Tumours Haematopoietic and Lymphoid Tissues. World Health Organization Classification of Tumours. IARC Press: Lyon, France, 2001, pp 162–167.
- 2 Hockenbery D, Nunez G, Millman C, *et al.* Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990;348:334–336.
- 3 Guo Y, Karube K, Kawano R, *et al.* Low-grade follicular lymphoma with t(14;18) presents a homogeneous disease entity otherwise the rest comprises minor groups of heterogeneous disease entities with Bcl2 amplification, Bcl6 translocation or other gene aberrances. *Leukemia* 2005;19:1058–1063.
- 4 Jardin F, Gaulard P, Buchonnet G, *et al.* Follicular lymphoma without t(14;18) and with

BCL-6 rearrangement: a lymphoma subtype with distinct pathological, molecular and clinical characteristics. *Leukemia* 2002;16:2309–2317.

- 5 Bosga-Bouwer AG, van den Berg A, Haralambieva E, *et al.* Molecular, cytogenetic, and immunophenotypic characterization of follicular lymphoma grade 3B; a separate entity or part of the spectrum of diffuse large B-cell lymphoma or follicular lymphoma? *Hum Pathol* 2006;37:528–533.
- 6 Bosga-Bouwer AG, van Imhoff GW, Boonstra R, *et al.* Follicular lymphoma grade 3B includes 3 cytogenetically defined subgroups with primary t(14;18), 3q27, or other translocations: t(14;18) and 3q27 are mutually exclusive. *Blood* 2003;101:1149–1154.
- 7 Katzenberger T, Ott G, Klein T, *et al.* Cytogenetic alterations affecting BCL6 are predominantly found in follicular lymphomas grade 3B with a diffuse large B-cell component. *Am J Pathol* 2004;165:481–490.
- 8 Ott G, Katzenberger T, Lohr A, *et al.* Cytomorphologic, immunohistochemical, and cytogenetic profiles of follicular lymphoma: 2 types of follicular lymphoma grade 3. *Blood* 2002;99:3806–3812.
- 9 Horsman DE, Okamoto I, Ludkovski O, *et al.* Follicular lymphoma lacking the t(14;18)(q32;q21): identification of two disease subtypes. *Br J Haematol* 2003;120:424–433.
- 10 Tagawa H, Karube K, Guo Y, *et al.* Trisomy 3 is a specific genomic aberration of t(14;18) negative follicular lymphoma. *Leukemia* 2007 21:2549–2551.
- 11 Karube K, Guo Y, Suzumiya J, *et al.* CD10-MUM1+ follicular lymphoma lacks BCL2 gene translocation and shows characteristic biologic and clinical features. *Blood* 2007;109:3076–3079.
- 12 Ohshima K, Kawasaki C, Muta H, *et al.* CD10 and Bcl10 expression in diffuse large B-cell lymphoma: CD10 is a marker of improved prognosis. *Histopathology* 2001;39:156–162.
- 13 Kodama T, Ohshima K, Nomura K, *et al.* Lymphomatous polyposis of the gastrointestinal tract, including mantle cell lymphoma, follicular lymphoma and mucosa-associated lymphoid tissue lymphoma. *Histopathology* 2005;47:467–478.
- 14 Eshoa C, Perkins S, Kampalath B, *et al.* Decreased CD10 expression in grade III and in interfollicular infiltrates of follicular lymphomas. *Am J Clin Pathol* 2001;115:862–867.
- 15 Tsujimoto Y, Ikegaki N, Croce CM. Characterization of the protein product of bcl-2, the gene involved in human follicular lymphoma. *Oncogene* 1987;2:3–7.
- 16 Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. *Annu Rev Immunol* 1998;16:395–419.
- 17 Sekiguchi N, Kobayashi Y, Yokota Y, *et al.* Follicular lymphoma subgrouping by fluorescence *in situ* hybridization analysis. *Cancer Sci* 2005;96:77–82.
- 18 Ruminy P, Jardin F, Picquenot JM, *et al.* Two patterns of chromosomal breakpoint locations on the immunoglobulin heavy-chain locus in B-cell lymphomas with t(3;14)(q27;q32): relevance to histology. *Oncogene* 2006;25:4947–4954.
- 19 Jardin F, Ruminy P, Bastard C, *et al.* The BCL6 proto-oncogene: a leading role during germinal center development and lymphomagenesis. *Pathol Biol (Paris)* 2007;55:73–83.

Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)