"Minor" *BCL2* Breakpoints in Follicular Lymphoma

Frequency and Correlation with Grade and Disease Presentation in 236 Cases

Olga K. Weinberg,* Weiyun Z. Ai,[†]
M. Rajan Mariappan,* Carol Shum,* Ronald Levy,[†]
and Daniel A. Arber*

From the Departments of Pathology* and Oncology,[†] Stanford University, Stanford, California

Follicular lymphomas are frequently associated with the t(14;18)(q32;q21). This translocation can be detected by karyotype, polymerase chain reaction (PCR), and fluorescence *in situ* hybridization (FISH). In addition to the breakpoints currently used for diagnosis located in the major breakpoint region (MBR) and the minor cluster region (mcr), recent studies have reported the existence of other breakpoints (3' *BCL2*, 5' mcr, and icr). In this study, we examined the frequency of all five breakpoints in 236 cases of follicular lymphomas by real-time PCR analysis. The distribution of breakpoint sites consisted of MBR in 118 cases (50%), mcr in 11 (5%), icr in 32 (13%), 3' *BCL2* in 13 (6%), and 5' mcr in three cases (1%). These findings illustrate significantly higher frequency of the icr breakpoint as compared with the more frequently studied mcr. Correlation of breakpoints with histology showed that MBR breakpoints occur more frequently in grade 2 lymphomas ($P = 0.042$). A majority of the PCR-negative cases (75%) contained an *IGH/BCL2* translocation with FISH methods, suggesting the presence of other *BCL2* breakpoints. Correlation of breakpoints with survival did not reveal significant differences. Diagnostic laboratories should consider expanding their PCR methods to include other *BCL2* breakpoints and correlating with FISH methods when appropriate. (*J Mol Diagn* 2007, 9:530–537; DOI: 10.2353/jmoldx.2007.070038)

Follicular lymphoma is the most prevalent form of low-grade B-cell lymphoma in adults. Although generally characterized by an indolent clinical behavior, many cases eventually transform to an aggressive large B cell lymphoma.¹ Derived from germinal center B cells, it is closely associated with the t(14;18)(q32;q21), involving *IGH* at 14q32 and *BCL2* at 18q21. A consequence of this translocation is an overexpression of anti-apoptotic protein *Bcl2*, which most likely represents the initial step of malignant transformation, leading to suppression of

apoptosis and progression to lymphoma.^{2,3} The *IGH/BCL2* rearrangement, although present in some diffuse large B-cell lymphomas, is a relatively specific molecular marker of follicular lymphoma and has been used for diagnostic and monitoring purposes.^{4–6}

In recent years, several studies have analyzed t(14;18) and *BCL2* breakpoints in follicular lymphomas with varying results.^{7–10} Although some differences in the rate of detection may be attributed to variations in histological diagnosis, the absence of standardized primers used and breakpoints studied among diagnostic laboratories may also be a factor. The t(14;18) has traditionally been detected by karyotype or Southern blot analysis, but the polymerase chain reaction (PCR) has more recently become the standard means of detection.^{7–9} In the past, standard PCR detection methods were based on the observation that most breakpoints on chromosome 18 cluster at two sites: in a 150-bp region in the 3' noncoding portion of the third exon of the *BCL2* gene, labeled as the major breakpoint region (MBR), and the 20 to 30-kb downstream region, known as the minor cluster region (mcr).^{7,8} On chromosome 14, the site of the breakpoints is the joining region (JH) of the immunoglobulin heavy chain. Others have shown the presence of breakpoints on chromosome 18, located between MBR and mcr, referred to as the intermediate cluster region (icr),¹⁰ and Buchonnet and colleagues¹¹ and others^{10,12,13} reported additional breakpoint clusters, referred to as 3' *BCL2* and 5' mcr. These various breakpoints are mapped in Figure 1.

To characterize further the frequency and distribution of *BCL2* breakpoints, Buchonnet and colleagues¹⁴ examined 113 untreated patients with t(14;18)-positive follicular lymphoma and found 65% to contain the MBR breakpoint, 12% to contain the 3' *BCL2* breakpoints, 8% with the 5' mcr breakpoints, and 9% with mcr breakpoint. These data illustrated that the 3' *BCL2* and 5' mcr break-

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R.L. is an American Cancer Society clinical research professor.

Address reprint requests to Olga Weinberg, M.D., Stanford University, Department of Pathology, 300 Pasteur Dr., Room L235, Stanford, CA 94305. E-mail: okw@stanford.edu.

Figure 1. A map of breakpoint sites in relation to the *IGH/BCL2* translocation.

points occur as frequently as mcr breakpoints in their population. In addition, 5' mcr cases were found to associate with bulky and high-stage disease and frequent extranodal involvement.¹⁴ Similarly, a smaller study by Batstone and Goodlad¹⁵ found that icr breakpoints occur as frequently as mcr breakpoints. However, the biological and clinical significance of these differences in *BCL2* rearrangement sites in follicular lymphomas remain unclear. Lopez-Guillermo and colleagues¹⁶ reported *BCL2* translocations to be an important prognostic factor using MBR and mcr breakpoints, whereas Montoto and colleagues¹⁷ found that *BCL2/IGH* rearrangements did not predict complete response to therapy or overall survival.

To substantiate further the frequency of minor breakpoints and their clinical significance, a large study comparing all new breakpoints (icr, 5' mcr, and 3' BCL2) with previously described MBR and mcr breakpoints is needed. In addition, there has not been an extensive search for correlations between the recently discovered breakpoints, histological subtypes of follicular lymphomas, and clinical presentation of the disease. The aim of this study is to determine the frequency of all breakpoints occurring in follicular lymphomas, evaluate negative cases with fluorescence *in situ* hybridization (FISH) for *BCL2* and *BCL6* translocations, and correlate the breakpoints with histological grade and site of disease. In addition, correlation of breakpoints with available clinical characteristics, response to therapy, and overall survival is evaluated on a subset of patients.

Materials and Methods

Materials

Two hundred thirty-six cases of follicular lymphoma with available frozen tissue were selected from the hematology laboratory at Stanford University Medical Center Stanford, Palo Alto, CA. All collected cases were received at Stanford between 1984 and 1998. Frozen tissue was verified with flow cytometry, histology, and/or immunohistochemistry for presence of follicular lymphoma at the time of tissue banking. Material for histological grading of the follicular lymphomas was available for 214 cases. Grading was performed using the criteria of the World Health Organization classification.¹⁸ The lymphomas were graded independently by two pathologists (D.A.A. and M.R.M.) and discrepant cases were reviewed to arrive at a consensus grade.

Source of DNA Sample

Genomic DNA was extracted from OCT-embedded frozen tissue samples using the DNeasy tissue kit animal tissue protocol (Qiagen, Valencia, CA).

Design of Quantitative PCR Assays

Primers and probes for the t(14;18) real-time PCR assays were designed using Primer Express Software v1.0 (Applied Biosystems, Foster City, CA) (Table 1). When possible, the dGTP/dCTP content of oligos did not exceed 40 to 70%, melting temperatures (*T_m*) of primer pairs were comparable and ~10°C greater than the *T_m* of the corresponding probe, and the probes 5' end did not contain dGTPs. All oligonucleotide primers and probes were synthesized by Operon Biotechnologies, Inc. (Alameda, CA). The probes were labeled with 6-carboxy fluorescein (FAM) at their 5' end and 6-carboxy-tetramethyl rhodamine (TAMRA) at their 3' end. Amplifiability of DNA was assessed through a real-time β -actin assay using primers and probe from the TaqMan β -actin detection reagents (Applied Biosystems).

Table 1. Primers and Probes Used for Real-Time PCR Assays on All 236 Samples

Gene segment	Real-time PCR primers, sequence 5' to 3'
MBR	5'-TTAGAGAGTTGCTTTACGTGGCC-3'
3' BCL2	5'-CCCAATAGGTGGAGGTGCA-3'
icr	5'-TGCAGAATCTGACGTTTCAGTCA-3'
MCR	5'-CCTGGCTTCCTTCCCTCTGT-3'
5' mcr	5'-AATGGAGCTGGAACAACCTGG-3'
JH	5'-GACCTGAGGAGACGGTGACC-3'
Fluorescent internal probes labeled at the 5' end with the reporter dye FAM and at the 3' end with the quencher dye TAMRA sequence 5' to 3'	
MBR	5'-FAM-TTTCAACACAGACCCACCCAGAGCC-TAMRA-3'
3' BCL2	5'-FAM-TGAGCTGAGATCACACCATTTGTACTCCAGC-TAMRA-3'
icr	5'-FAM-CTGAAATATGACCAAAATGGAAACCTCCCC-TAMRA-3'
MCR	5'-FAM-TCTCTGGGGAGGAGTGGAAGGAAGG-TAMRA-3'
5' mcr	5'-FAM-TGGATCAGAAATGAATGCCATCTCAA-TAMRA-3'

Table 2. Distribution of Breakpoints in 236 Cases of Follicular Lymphomas

Breakpoint category	No. of cases	Percent
MBR	118	50
icr	32	13
3' BCL2	13	6
mcr	11	5
5' mcr	3	1
MBR/icr	5	2
MBR/3' BCL2	2	1
MBR/5' mcr	1	0
Negative	51	22
Total	236	100

Polymerase Chain Reaction Amplification

Each reaction contained 500 ng of genomic DNA, 50 pmol of each primer, and 20 pmol of the corresponding probe. The standard master mix was composed of reagents obtained from Applied Biosystems and included 10% TaqMan 10× PCR Gold buffer; 2.5 mmol/L MgCl₂; 0.2 mol/L of dATP, dCTP, dGTP, and dTTP; and 1.5 U of AmpliTaq Gold DNA polymerase. All reactions were brought to a 50-μl volume using sterile H₂O. Reactions were placed in MicroAmp optical 96-well reaction plates and covered with optical adhesive covers. The reaction conditions were 10 minutes at 95°C (activation and predenaturation) followed by 45 cycles of 15 seconds at 95°C (denaturation) and 1 minute at 60°C (combined annealing/extension). Fluorescence data were collected during the annealing/extension phase of every cycle, using the ABI Prism 7700 sequence detection system containing a 96-well thermal cycler (Applied Biosystems). All 236 samples were tested with primers shown in Table 1.

FISH Studies

The *IGH/BCL2* dual-color, dual-fusion translocation probe from Vysis (Downers Grove, IL) was hybridized to detect t(14;18) on touch preparations from frozen tissue samples. The *IGH* probe spans ~1.5 Mb and contains sequences homologous to essentially the entire *IGH* locus, as well as sequences extending ~300 kb beyond the 3' end of the *IGH* locus. The LSI *BCL2* probe covers an ~750-kb region, including the entire *BCL2* gene with additional sequences extending ~250 kb both distal and proximal to the gene. The *BCL6* probe (Vysis) is a dual-color, break-apart rearrangement probe and consists of a

mixture of a 300-kb labeled Spectrum Orange 5' LSI *BCL6* probe and a 600-kb labeled Spectrum Green 3' LSI *BCL6* probe. These two probes are separated by a 42-kb gap that contains the entire *BCL6* gene, including the *BCL6* breakpoint region. FISH analysis was performed on residual frozen tissue with appropriate controls, following the manufacturer's instructions.

Clinical Data

Clinical survival data were available for evaluation on 149 patients. Overall survival was calculated from time of diagnosis to date of death or last follow-up date for living patients. Survival curves were plotted using Kaplan-Meier methods. Clinical characteristics and response to therapy were available in 62 patients. The Follicular Lymphoma International Prognostic Index (FLIPI) was determined using age, Ann Arbor stage, hemoglobin, number of nodal areas involved, and serum lactate dehydrogenase level.

Statistical Analysis

Data were analyzed using the χ^2 test with Yates correction.

Results

Frequency of BCL2 Breakpoints

Two hundred thirty-six cases with histologically confirmed follicular lymphoma were studied for the five *IGH/BCL2* translocation breakpoints using real-time PCR and primers shown in Table 1. Figure 1 illustrates the location of the breakpoints on chromosome 18 and the expected product size for each breakpoint. In total, 185 cases contained a *BCL2* translocation using this method (78% of all cases). Screening for translocations in *BCL2* using MBR and mcr primers demonstrated breakpoints at MBR in 118 patients (50%) and mcr in 11 patients (5%) (Table 2). The distribution of other minor breakpoints was as follows: icr in 32 patients (13%), 3' BCL2 in 13 patients (6%), and 5' mcr in three patients (1%). Double breakpoints were found in eight cases, and all of these cases contained an MBR breakpoint along with icr in five patients (2%), 3' BCL2 in two (1%), and 5' mcr in one patient (Table 2). The remaining 51 cases were negative for any

Table 3. Correlation of BCL2 Breakpoints with Histological Grade in 170 Follicular Lymphomas

	Grade 1	Grade 2	Grade 3	P value
MBR	43 (63%)	47 (80%)	22 (61%)	0.042
icr	17 (25%)	6 (10%)	8 (22%)	0.014
3' BCL2	4 (6%)	5 (8%)	4 (11%)	0.65
mcr	5 (7%)	3 (5%)	3 (8%)	0.81
5' mcr	2 (3%)	1 (2%)	0 (0%)	0.37
Total	68*	59*	36*	

*Cases with double breakpoints subtracted from total.

Table 4. Cases without PCR Detectable Breakpoints and Correlation with Grade

PCR ⁻	Grade 1	Grade 2	Grade 3
FISH BCL2 ⁺	13	13	11
FISH BCL6 ⁺	2	0	4
FISH ⁻	5	1	2
Total	20	14	17

The distribution of grades in PCR-negative cases is not significant ($P > 0.05$).

breakpoints by the above-described real-time PCR techniques.

FISH Analysis for BCL2 and BCL6 Translocations

The 51 cases found negative by PCR for any breakpoints were tested for *IGH/BCL2* translocations with FISH using residual frozen tissue. Observation of two distinct signals of each color, green and orange, represents a normal hybridization pattern. The presence of a fusion was indicated by a yellow signal, which represents the derivative chromosome. Cases with more than 20% cells with yellow signal were considered as positive, whereas negative controls showed less than 20% cells with yellow signal. Of the 51 follicular lymphomas found to be negative with PCR methods, 37 cases were found to contain the *IGH/BCL2* translocation with FISH (Table 4). The remaining 14 cases were evaluated with FISH for *BCL6* translocations. Six cases were found to contain *BCL6* translocations by FISH using break-apart probes and by counting cases with more than 15% cells with separated orange and green signal as positive. Eight cases (from a total of 236 or 3%) were negative for both the *BCL2* and *BCL6* translocations using both PCR and FISH methods.

Correlation of Breakpoints with Histological Grade

The overall distribution of histological grades in the 214 patient cases studied consisted of 88 cases of grade 1 (41%), 73 of grade 2 (34%), and 53 of grade 3 (25%) follicular lymphomas. Table 3 summarizes the frequency of all breakpoints for each histological grade but excludes the eight cases with double breakpoints from the total count. We found significant correlation between histological grade and the presence of MBR and icr breakpoints. Specifically, breakpoints in the MBR cluster oc-

curred more frequently in grade 2 lymphomas compared with grades 1 and 3 ($P = 0.042$). However, grade 2 follicular lymphomas were also associated with a significantly lower frequency of the icr breakpoint ($P = 0.014$). No correlation between 3' BCL2, 5' mcr, and mcr and histological grade was found, although the total number of these breakpoints was small (Table 3).

Detection of *IGH/BCL2* rearrangements by FISH was correlated with histological subtypes. In the 37 PCR breakpoint-negative cases, which all contained the *IGH/BCL2* translocations using the FISH method, there were 13 cases of grade 1, 13 cases of grade 2, and 11 cases of grade 3 follicular lymphomas (Table 4). Cases containing the *BCL6* translocation were associated with grade 3 disease (four cases of six total). Furthermore, in the eight remaining cases, negative for both *IGH/BCL2* and *BCL6* translocations, a higher proportion of patients (five cases or 62%) contained grade 1 follicular lymphomas as compared to grades 2 and grade 3 (one and two cases, respectively), but this did not reach statistical significance ($P = 0.06$).

Correlation with Extranodal and Supra- and/or Subdiaphragmatic Disease

Extranodal disease, as defined at the time of diagnostic biopsy, was present in 33 patients (sites included stomach, thyroid, tongue, parotid, spleen, neck, skin, and eye) and comprised 14% of the total cases examined. A significant correlation of translocation breakpoints with extranodal disease could not be made (Table 5). However, it is interesting that five cases of the extranodal disease contained the icr breakpoint (15% of all extranodal cases), and 18 contained the MBR breakpoint (54%). Furthermore, seven extranodal cases (22%) were negative for all of the major breakpoints using PCR techniques, but six of these cases in fact contained the *IGH/BCL2* translocation using the FISH technique, suggesting other breakpoints in these cases. The distribution of breakpoints in the nodal disease was similar to the overall distribution with icr second in frequency after MBR (Table 5).

The studied cases of follicular lymphoma with available information were divided into supradiaphragmatic and subdiaphragmatic disease, as evident at time of diagnosis. At the time of presentation, subdiaphragmatic disease occurred in 83 cases and supradiaphragmatic disease in 72 cases (Table 5). We found a significant correlation of breakpoints at icr with subdiaphragmatic

Table 5. Correlation of Breakpoints with Presentation of Disease

	Nodal	Extranodal	Supradiaphragmatic	Subdiaphragmatic
MBR	94	18	53	50
icr	26	5	7*	21
3' BCL2	11	2	7	5
mcr	9	2	5	4
5' mcr	3	0	0	3
Total	143	27	72	83

*Comparing supra- and subdiaphragmatic disease, $P = 0.041$ for the icr breakpoint, all others $P > 0.05$.

Table 6. Correlation of Breakpoints with Available Clinical Data on 149 Patients

	Number of patients	MBR (n = 83)	Minor breakpoints (n = 54)	No detectable breakpoints (n = 12)
Age at diagnosis	149	44	44	41
B symptoms:				
Present	14	7 (9%)	6 (12%)	1 (10%)
Absent	125	71 (91%)	45 (88%)	9 (90%)
Stage				
I or II	9	4 (5%)	3 (6%)	2 (20%)
III	42	26 (33%)	15 (29%)	1 (10%)
IV	88	48 (62%)	33 (65%)	7 (70%)
Overall survival (years)	149	12.5	11.4	12.1

P values obtained in comparing B symptoms, stage and overall survival are not statistically significant ($P > 0.05$). Number of patients with available clinical data, including B symptoms, stage, and survival data, is less than the number of patients sub-grouped as MBR, minor, or lack of detectable breakpoints (given as *n*).

diseases ($P = 0.041$); 21 cases of subdiaphragmatic disease contained the icr breakpoint as compared to only seven cases of supradiaphragmatic disease.

Correlation of Breakpoints with Prognosis and Response to Therapy

Clinical parameters were available on 149 patients and are summarized in Table 6. To simplify analysis, patients were sorted according to their breakpoint sites into three separate groups: MBR, minor breakpoints, and a breakpoint-negative. Minor breakpoints included mcr, icr, 3' BCL2, and 5' mcr, as well as evidence of *IGH/BCL2* fusion as demonstrated by the FISH method. Overall, 83 patients contained breakpoints at the MBR cluster, and 54 patients contained minor breakpoints whereas the breakpoint-negative group consisted of 12 patients. As shown in Table 7, clinical presentation was similar in all three groups, with no significant differences in age, B symptoms, and stage. The median age at time of diagnosis was 45, with a range between 23 to 72 years of age. Most of the patients presented with stage III or IV disease. At the time of presentation, B symptoms were noted

in seven patients with MBR breakpoints (9%) compared with six patients (12%) with minor breakpoints, and in one patient without any detectable breakpoints (10%). The median overall survival time of all three groups was comparable, with 12.5 years for patients with major breakpoints, 11.5 years for patients with minor breakpoints, and 12.1 years in the breakpoint-negative group (Figure 2). Overall, 78 of 149 patients were deceased at the time of current analysis.

Thorough clinical follow-up data were available on 62 patients and were analyzed to evaluate prognostic features of the breakpoints. In this group of patients, the breakpoint site occurred at MBR in 32 patients, at the mcr in six, at icr in seven, 3' BCL2 in three, and 5' mcr in two patients. In addition, eight patients did not contain any detectable breakpoints with PCR analysis but were demonstrated to have t(14;18) translocation by FISH method. The remaining five patients did not have any *BCL2* breakpoints with either PCR or FISH analysis and were thus regarded as breakpoint-negative cases. Patients with breakpoints located in the mcr, icr, 3' BCL2, and 5' mcr regions, as well as *IGH/BCL2* FISH-positive cases were considered as minor breakpoints (Table 7).

Table 7. Correlation of Breakpoints with Available Clinical Data on 62 Patients

	Number of patients	MBR	Minor breakpoints (n = 24)	No detectable breakpoints (n = 5)
Age	62	44	44	41
B symptoms	62	9 (28%)	3 (11%)	0
Stage				
III	62	10 (31%)	9 (35%)	1 (20%)
IV	62	22 (69%)	17 (65%)	4 (80%)
FLIPI				
High	10	7 (28%)	2 (10%)	1 (25%)
Intermediate	35	16 (64%)	16 (80%)	3 (75%)
Low	4	2 (8%)	2 (10%)	0 (0%)
Number of patients transformed	63	3 (9%)	3 (11%)	0 (0%)
Time from diagnosis to treatment (days)	63	168	121	761
Response to CVP				
CR	9	4 (21%)	5 (50%)	1 (100%)
<CR	20	15 (79%)	(50%)	0

P values obtained in comparing B symptoms, stage, FLIPI, and response to CVP are not statistically significant ($P > 0.05$). Number of patients with available clinical data, including B symptoms, stage, FLIPI score, time from diagnosis to treatment, and response to CVP is less than the number of patients sub-grouped as MBR, minor, or lack of detectable breakpoints (given as *n*).

Figure 2. Survival curve of 149 patients with follicular lymphomas. Patients were divided into three groups according to the site of their *BCL2* breakpoints. Minor breakpoints consisted of mcr, icr, 3' *BCL2*, and 5' mcr, as well as PCR-negative cases that tested positive with FISH for *IGH/BCL2* fusion. Negative breakpoint group consisted of cases that tested negative by both PCR and FISH methods. The overall survival in three groups was comparable.

Clinical presentation in these 62 patients was comparable, with most patients presenting with stage III or IV disease (30 and 70%, respectively, for the total group). Furthermore, the average age at time of diagnosis was similar, ranging between 26 and 50 years of age, with a median age of 44. However, B symptoms, as defined by fevers, night sweats, and weight loss, occurred in only 12 patients, which is 19% of the total patients followed in this group. The majority of these patients with B symptoms (9 of 12 or 75%) were found to contain breakpoints in the MBR region (Table 7). Interestingly, patients without detectable breakpoints in this group did not present with B symptoms. However, this finding did not reach statistical significance.

The FLIPI score was determined in 49 patients and categorized into high-, intermediate-, or low-risk groups.¹⁹ Overall, 10 patients (or 20%) were found to have a high FLIPI risk score, 35 an intermediate score (71%), and four with a low scores (8%). Of the patients with a high FLIPI score, seven contained breakpoints in the MBR cluster (70%), one in the icr region, and two did not contain breakpoints by PCR but were confirmed to contain *BCL2* or *BCL6* translocations by FISH analysis. Although different in all three groups, the distribution of FLIPI scores did not significantly correlate with breakpoints.

Transformation to diffuse large cell lymphoma occurred in six patients. Three of these patients contained breakpoints in the MBR cluster and one in the mcr region. The remaining two patients did not have breakpoints by PCR analysis but did contain *BCL2* and *BCL6* translocation. In addition, median time from diagnosis to first treatment, an indicator of more aggressive disease, was found to be lower in patients with MBR and minor breakpoints as compared to those without detectable breakpoints; however, this finding was not statistically significant.

Discussion

Follicular lymphomas are characterized by the presence of the t(14;18)(q32;q21), which causes a fusion of the *BCL2* oncogene with the immunoglobulin heavy chain joining region. The *IGH/BCL2* rearrangement is a rela-

tively specific molecular marker of follicular lymphoma, frequently used for diagnosis and monitoring of disease.^{5,6} The most commonly used primers to detect the *IGH/BCL2* rearrangement target the MBR and mcr breakpoints. However, previous studies have shown that these primers can fail to detect up to 30% of follicular lymphoma cases¹⁰⁻¹⁴; we found that these primers failed in 42% of our 236 cases.

Different studies have now reported new breakpoints that localize within the commonly analyzed clusters.¹⁰⁻¹³ In the largest study to date, Buchonnet and colleagues¹⁴ found that unusual breakpoints occur in 20% of follicular lymphomas. However, the icr breakpoint was not included in their study. More recent studies have indicated that breakpoints at the icr cluster occur at least as frequently as mcr.¹⁵ A study of BIOMED2 primers by van Dongen and colleagues,²⁰ using a two-tube multiplex system, evaluated breakpoints within the non-MBR region and found that up to 8% of cases had breakpoints in 3' MBR and 4% in 5' mcr cluster. We investigated the frequency and clinical significance of the icr breakpoint and found that, after MBR, icr was the most frequent breakpoint. Breakpoints at the icr cluster comprised 13% of our cases, significantly higher than the currently clinically used mcr breakpoint, noted to be present in only 5% of our cases. The other minor breakpoint, 3' *BCL2*, occurred less often than icr but more frequently than the mcr breakpoint. In summary, our study showed that minor breakpoints, as detected by PCR, occurred in 23% of follicular lymphomas.

The remaining 51 PCR-negative cases were evaluated with FISH methods and found to contain the *IGH/BCL2* translocation in 37 cases (72%). This illustrates that FISH is a useful test in the diagnosis of follicular lymphomas, consistent with literature reports,²¹⁻²⁴ and will detect cases that even a comprehensive PCR approach will miss. Furthermore, this finding also suggests the presence of other novel, undetected breakpoints. The combined PCR and FISH methods for *IGH/BCL2* translocations lead to only 14 truly negative cases (6%).

Six of the 14 *IGH/BCL2*-negative cases were found to contain *BCL6* translocations by FISH. Although *BCL6* is a relatively rare translocation in low-grade follicular lymphomas, it has been noted as a frequent gene alteration in grade 3 follicular lymphomas.²² Four cases of the six found to contain *BCL6* translocations in this study consisted of grade 3 follicular lymphomas (Table 3). The remaining eight negative cases consisted of mostly grade 1 and 2 diseases (five and one case, respectively). This distribution of histological grades contradicts prior reports, which have associated lack of *BCL2* rearrangement, as assessed by molecular breakpoint analysis, with high-grade disease.²⁵⁻²⁷ However, in a study of 54 cases of t(14;18)-negative follicular lymphomas, Horsman and colleagues¹ showed that the distribution of histological grades was similar to this study, with 42% of grade 1 disease, 40% of grade 2, and 18% of grade 3 disease.

The clinical significance of *BCL2* breakpoints continues to remain unclear despite numerous studies.^{14,16,17} We looked for correlations between breakpoints and histological grade. The overall histological distribution of our

cases is similar to that reported by Guo and colleagues.²⁸ A statistically significant correlation between grade and breakpoints was found for breakpoints at the MBR and icr clusters, with more frequent number of MBR and icr breakpoints occurring in grade 2 follicular lymphoma cases. Furthermore, the icr breakpoint was found to be more significantly associated with subdiaphragmatic disease.

To evaluate further the clinical significance of *BCL2* breakpoints, available clinical follow-up data on a subset of patients were examined. Analysis of these data showed that B symptoms tend to occur more frequently in patients with breakpoints in the MBR cluster. Furthermore, patients with breakpoints in the MBR cluster also tended to fall more frequently into high FLIPI risk group as compared to patients with minor breakpoints. As expected from the high FLIPI score, most patients with this breakpoint did not reach complete response to therapy. In addition, three of six patients with transformed disease contained breakpoints in the MBR region, providing further evidence for possible clinical importance of this breakpoint. However, these findings did not reach statistical significance, although the statistical analysis was limited by both the total number of available cases and fewer number of patients with minor breakpoints. In looking at overall survival for a larger group of the patients, we were unable to show a survival difference when comparing patients with MBR breakpoints to the patients with the other less common breakpoints. Similarly, B symptoms did not seem to significantly correlate with breakpoints in a larger group of patients.

In 33 of the 236 cases (14%), the follicular lymphomas were extranodal at the time of diagnosis. The distribution of breakpoints in the extranodal cases was similar to that of the nodal cases (eg, the icr breakpoint is the second most frequently found breakpoint after MBR). Thus, in addition to possessing a similar morphology and immunophenotype,²⁶ extranodal and nodal follicular lymphomas have a similar breakpoint signature. The *IGH/BCL2* translocation was found in 32 of our extranodal cases (97%), contrary to the findings of Goodlad and colleagues,^{29,30} who found this translocation in only ~15% of extranodal cases in two separate studies. This difference in presence of *BCL2* translocations could be analogous to variations among studies in the frequency of t(14;18) in primary cutaneous follicular lymphoma. In that extranodal site, there is a suggestion that the frequency of the translocation may be geographical, occurring more commonly in North American studies than in European studies.³¹⁻³⁵

In summary, this study emphasizes the importance of including minor breakpoints, especially ones located at the icr cluster, in PCR diagnostic techniques of follicular lymphomas. Furthermore, FISH is a useful technique to confirm *BCL2* and *BCL6* translocations in PCR-negative cases. In addition to correlating with grade 2 follicular lymphomas, breakpoints at the icr region were significantly associated with subdiaphragmatic disease. This suggests a possible biological importance of the icr breakpoint and implies the clinical necessity of including it in diagnosis and monitoring purposes. Although no

significant differences in outcome based on breakpoints were identified in this study, the detection of a t(14;18) is useful for the diagnosis and monitoring of patients, and an expanded PCR approach significantly increases this detection rate.

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