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Biomarkers for Risk Stratification in Patients with Previously Untreated Follicular Lymphoma Receiving Anti-CD20-Based Biologic Therapy

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Abstract

Follicular lymphoma (FL) is an indolent B-cell neoplasm of germinal center origin. Standard treatment regimens consist of anti-CD20 therapy with or without chemotherapy. While high response rates to initial therapy are common, patients ultimately relapse or have progressive disease. Clinical risk factors such as the FL international prognostic index (FLIPI) have been

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identified, but there is need for prognostic and predictive biomarkers. We studied markers of lymphoma cells and tumor microenvironment by immunohistochemistry in tissue samples from patients enrolled in one of four phase 2 trials of anti-CD20-based biologic therapy for previously untreated grades 1-2 or 3A FL. Results were correlated with progression-free survival (PFS) and PFS status at 24 months (PFS24). The four trials included 238 patients (51.1% male, median age 55 years) with stage III, IV, or bulky stage II disease. By FLIPI, 24.6% had low-risk, 56.8% had intermediate-risk and 18.6% had high-risk disease. Outcome differed significantly for patients treated with lenalidomide and rituximab (CALGB 50803) compared to the other three trials (median PFS not reached vs. 3.0 years, HR 3.47, 95% CI 2.11-5.72); therefore, data were stratified by clinical trial (CALGB 50803 vs. all others) and adjusted for FLIPI risk group. Among 154 patients with available tissue, interfollicular BCL6 positivity, interfollicular CD10 positivity, and elevated Ki67 proliferation index >/=30% within neoplastic follicles were each associated with inferior PFS and a high risk of early event by PFS24. We identify promising biomarkers for FL risk stratification that warrant further validation in phase 3 trials.

Keywords

follicular lymphoma; BCL6; CD10; Ki67 proliferation index; prognostic biomarkers

Introduction

Follicular lymphoma is an indolent B-cell neoplasm of germinal center origin associated with upregulation of the anti-apoptotic BCL2 protein via the t(14;18)/*IGH-BCL2* translocation in most cases. Standard first-line treatment regimens in symptomatic patients with advanced-stage disease typically consist of anti-CD20 monoclonal antibody therapy with or without chemotherapy.[1] The latter is usually reserved for advanced-stage patients with high tumor burden. Immunodulatory agents, such as lenalidomide, are also an option in the upfront setting.[2] While high response rates are common, relapses and progression ultimately occur. Clinical risk factors such as the follicular lymphoma international prognostic index (FLIPI and FLIPI2),[3, 4] as well as a clinicogenetic risk model (m7-FLIPI),[5] have been identified, but there is further need for practical prognostic and predictive biomarkers to aid in risk stratification and development of novel treatment strategies.[6]

Biomarkers of interest that have been identified previously by immunohistochemistry include both tumor cell and microenvironmental immune markers. Among tumor cell markers, elevated expression of the naïve B-cell marker FOXP1[7] and the post-germinal center antigen MUM1 (IRF4)[8, 9] have been associated with an unfavorable prognosis in separate studies, as has elevated Ki67 (MIB1) proliferation index within neoplastic follicles of histologically low-grade (grade 1-2 of 3) cases.[10] Despite the well-known patterns of expression of germinal center markers CD10 and BCL6, abnormal or heterogeneous expression occurs in different follicular lymphoma compartments.[11, 12] While some cases recapitulate the normal pattern of downregulation of CD10 and BCL6 in tumor cells in the interfollicular location, others abnormally retain expression.[13] Among microenvironmental immune markers, increased numbers of tumor-associated macrophages, as assessed by CD68 staining, has been associated with a shorter overall survival in several studies,[14-17] but

predicted for a favorable outcome in one study of patients treated with rituximab and chemotherapy.[18] Granzyme B, a cytotoxic T-cell marker, has been associated with a longer progression-free survival (PFS) when a high perifollicular content of granzyme B-positive cells is seen in tissue sections.[19] Results have been more variable for other T-cell subsets, such as number or density of PD1-positive T follicular helper cells within follicles[15, 20] and follicular vs. diffuse patterns of expression of FoxP3-positive T regulatory cells.[14, 16, 21] This variability in findings may be related to several factors such as the single institutional nature of some studies and heterogeneity in patient selection and therapy, as is common in follicular lymphoma.

We sought to evaluate biomarkers of both tumor cells and tumor-associated immune cells via a prospective, multicenter analysis using tissue collected from patients enrolled in one of four phase 2 Alliance for Clinical Trials in Oncology (formerly Cancer and Leukemia Group B [CALGB]) clinical trials of untreated follicular lymphoma patients receiving rituximab or similar biologic therapy.

Materials and Methods

Patients and Samples

Correlative studies were performed in tumor samples from patients enrolled in one of the following phase 2 Alliance clinical trials of biologic therapies in untreated grades 1-2 or 3A follicular lymphoma: (1) galiximab (anti-CD80 monoclonal antibody) and rituximab (CALGB 50402);[22] (2) extended induction epratuzumab (anti-CD22 monoclonal antibody) and rituximab (CALGB 50701);[23] (3) lenalidomide and rituximab (CALGB 50803);[24] and (4) ofatumumab (anti-CD20 monoclonal antibody with increased CD20 affinity and complement-dependent cytotoxicity) (CALGB 50901).[25] Each patient signed an IRB-approved, protocol-specific informed consent document for use of samples in accordance with federal and institutional guidelines. Enrollment in all four trials was restricted to patients with stage III, IV or bulky stage II (single mass >/=7 cm in greatest dimension) disease, while enrollment in CALGB 50803 and CALGB 50901 was additionally targeted to patients with low- and intermediate-risk disease by FLIPI; tumor burden (e.g. GELF) was not part of eligibility criteria. Given the overall similar eligibility criteria, study sizes, anti-CD20 monoclonal antibody-based treatment strategies, and median durations of follow-up, results of these four studies were analyzed in aggregate for the purposes of biomarker analysis.

Follicular lymphoma diagnosis and grade were histologically confirmed by central pathology review on whole slide sections. Histologic confirmation was performed independently by two pathologists, with a third reviewer if there was disagreement regarding diagnosis and/or grade (<5% of cases). Cases with grade 3B histology were excluded from further analysis. For immunohistochemical analysis, individual tissue microarrays (TMAs) were constructed separately for each of the four trials using 1 mm-diameter formalin-fixed paraffin-embedded tissue cores taken from diagnostic tumor samples from all patients with available tissue. Each case was sampled in duplicate from specific areas containing neoplastic follicles, as annotated by pathologists at the time of central review, resulting in an average of 3-6 follicles sampled per core (6-12 follicles per case). The validity of using

TMAs to study various immunohistochemical characteristics and immunoarchitectural features of follicular lymphoma has been previously established.[16, 26-28] Table 1 lists the tumor cell and microenvironmental markers that were selected for immunohistochemical analysis based on prior studies suggesting prognostic significance and the staining characteristics used in their evaluation. For tumor cell markers, we studied extent of expression of germinal center B-cell markers within and outside neoplastic follicles, as well as the percentage staining of tumor cells by post-germinal center, naïve B-cell, anti-apoptotic and proliferation markers. For tumor-associated immune cells, we studied both macrophages and various T-cell subsets. Specifically, for Ki67 we explored a cut-off of >/=30% staining within follicles, which previously identified a group of histologically low-grade follicular lymphomas with a high proliferation index associated with clinically aggressive disease.[10] The basis for cut-offs that were explored for other biomarkers are listed in Table 1. Immunohistochemistry was performed using standard laboratory methods and all stains were evaluated via consensus review among three hematopathologists (AC, JWS, EDH), except for Ki67, which was quantified in neoplastic follicles using image analysis software (Definiens, Carlsbad, CA).

Statistical Analysis

Data were collected and analyzed by the Alliance Statistics and Data Center per Alliance policies and procedures; data were locked on 3/9/2018 for CALGB 50701, 50803 and 50901 and on 3/13/2018 for CALGB 50402. Progression-free survival (PFS) and response were defined as per individual clinical trial protocol. PFS status at 24 months (PFS24) was used to define an early event per a previous publication from these clinical trials.[29] The primary analysis was the association between biomarkers and PFS/PFS24. Due to high response rates on the trials limiting the utility of response in analyses, associations between biomarkers and overall response rate were secondary. Associations between biomarkers and overall response were measured using cross-tabulations and Fisher's exact test. Associations between biomarkers and PFS were assessed using Cox proportional hazards models and reported via hazard ratios (HR) and 95% confidence intervals. Cox models were stratified by clinical trial (CALGB 50803 vs. all others) and adjusted for FLIPI risk group to address differences in PFS/PFS24 between clinical trials and adjust for differences in patient characteristics. Due to the observed confounding between staining and trial outcomes, in particular for BCL6 and CALGB 50803, graphical survival curves were adjusted for clinical trial and FLIPI as described by Therneau *et al* in the R survival package vignette.[30, 31] Associations between biomarkers and PFS24 were assessed using logistic regression models adjusted for clinical trial and FLIPI and reported via odds ratios (OR) and 95% confidence intervals. Any reported p-values and widths of 95% confidence intervals have not been adjusted for multiplicity. Data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC, USA) and R version 3.4.2.

Results

Patient Characteristics

The four trials included a total of 238 previously untreated patients (121 men, 51.1%) with a median age of 55 years (range 22-90). Patients were stratified by FLIPI risk groups as

follows: 58 (24.6%) low-risk, 134 (56.8%) intermediate-risk, and 44 (18.6%) high-risk disease (data missing for two patients). Baseline patient characteristics were similar across the four trials, except for patients enrolled in the ofatumumab trial (CALGB 50901) who were slightly older with more intermediate-risk disease (see Table, Supplemental Digital Content 1).

Overall, 154/238 patients (64.7%) had tissue available for biomarker analysis; characteristics of this subgroup are summarized in Table 2. Patients with available tissue had higher risk disease by FLIPI (score 3-5 20.4% vs. 15.5%) with higher stage (III-IV 96.7% vs. 89.3%) and bulkier disease (82.9% vs. 64.7%,) compared to those without available tissue (see Table, Supplemental Digital Content 2). There were no significant differences in other baseline characteristics between the two groups.

Because of the variability in tumor sampling and histological sectioning inherent to tissue microarrays, the actual number of cases available for analysis ranged from 119 to 148 depending on the stain being analyzed (Table 3). Patients undergoing treatment with lenalidomide and rituximab (CALGB 50803) and ofatumumab (CALGB 50901) showed a higher prevalence of interfollicular BCL6 positivity, while more cases in the ofatumumab trial (CALGB 50901) showed a higher Ki67 proliferation index within follicles. Staining results were otherwise similar across the four studies.

Biomarkers and Outcome

Median follow-up on the trials ranged from 2.6 to 5 years. The overall response rate was very high across all trials (range 79-95%) resulting in a limited endpoint for analyses (see Table, Supplemental Digital Content 3); therefore, the primary endpoints for outcome were PFS and PFS24. Patients receiving lenalidomide and rituximab (CALGB 50803) had significantly better PFS compared to patients enrolled in the other three trials (median PFS not reached vs. 3.0 years, HR 3.47, 95% CI 2.11-5.72, [see Figure, Supplemental Digital Content 4]). The superior PFS for patients receiving lenalidomide and rituximab was sustained when analysis was restricted to cases with tissue available for biomarker analysis (data not shown). Further details related to clinical outcomes are reported separately.[22-25] All analyses were thus adjusted for clinical trial (lenalidomide vs. no lenalidomide) and FLIPI.

BCL6 and CD10—Interfollicular BCL6 was positive in 53% of patients (Table 3, Figure 1A-B) and strongly associated with inferior PFS (HR=2.2, 95% CI: 1.35-3.58), as well as a high risk of early event by PFS24 (OR=5.25, 95% CI: 1.97-13.98) (Table 4). Adjusted survival curves for interfollicular BCL6 and PFS are shown in Figure 2A. Unadjusted PFS curves for interfollicular BCL6 by trial are shown in Supplemental Digital Content 5. Nearly all patients (95%) had follicular expression of BCL6, which precluded its relevance as a clinical biomarker.

Interfollicular CD10 was positive in 50% of patients (Table 3, Figure 1C-D) and showed a trend toward inferior PFS (HR=1.48, 95% CI: 0.94-2.34), but was associated with a high risk of early event by PFS24 (OR=2.60, 95% CI: 1.05-6.43) (Table 4). Adjusted survival curves for interfollicular CD10 and PFS are shown in Figure 2B. Nearly all patients (89%) had

follicular expression of CD10, which limited its relevance as a clinical biomarker, though follicular CD10 expression again showed a trend toward inferior outcome: PFS HR=1.82, 95% CI: 0.83-4.00; PFS24 OR=2.74, 95% CI: 0.66-11.42.

Given that both BCL6 and CD10 are germinal center B-cell markers and showed similar associations with PFS with an interfollicular pattern of staining, we also examined the effect of combined interfollicular positivity for BCL6 and CD10, which was seen in 44/126 patients (35%). This staining characteristic was strongly associated with inferior PFS (HR=2.50, 95% CI: 1.37-4.54) and a high risk of early event by PFS24 (OR=9.07, 95% CI: 2.36-34.78), with negative interfollicular staining for both BCL6 and CD10 as a reference. Adjusted survival curves for interfollicular BCL6 and CD10 co-expression and PFS are shown in Figure 2C. As can be seen from this figure, while there is a trend toward shorter PFS with interfollicular expression of either marker, it is the combined expression of BCL6 and CD10 outside of the follicle environment that is associated with shorter PFS.

Ki67—High Ki67 proliferation index of >/=30% within follicles was identified in 13% of patients (Table 3, Figure 3) and associated with inferior PFS (HR=2.47, 95% CI: 1.33-4.61), as well as a high risk of early event by PFS24 (OR=7.49, 95% CI: 1.77-31.73) (Table 4). Adjusted survival curves for Ki67 and PFS are shown in Figure 2D. Among the 17 cases with Ki67 >/=30%, 14 were grade 1-2 and three were grade 3A. Outcomes were similar when analysis was restricted to patients with grade 1-2 disease (N=117, including 103 cases with Ki67 <30% and 14 cases with Ki67 >/=30%): PFS HR=2.52, 95% CI: 1.25-5.08 (survival curves not shown); PFS24 OR=8.77, 95% CI: 1.61-47.75.

Granzyme B—Increased numbers of granzyme B-positive cytotoxic T cells in perifollicular areas at a level of >/=10% of all cells was seen in only 10% of patients (Table 3), but associated with inferior PFS (HR=2.45, 95% CI: 1.11-5.39), as well as a high risk of early event by PFS24 (OR=9.63, 95% CI: 1.00-92.12).

The remaining markers were either lacked sufficient variability (<10% or >90% positive) to be considered for outcome analysis (Blimp1, BCL2) or showed no meaningful association with PFS or PFS24 (MUM1, FOXP1, FOXP3, CD68, follicular and interfollicular PD1).

Discussion

In this study, we evaluated both tumor-associated and microenvironmental biomarkers by immunohistochemistry in follicular lymphoma patients enrolled in one of four Alliance clinical trials of rituximab or similar biologic therapy. We identify three adverse prognostic features related to tumor cells, high Ki67 proliferation index within follicles and interfollicular BCL6 and CD10 positivity, as well as one tumor-infiltrating lymphocyte (TIL)-related biomarker, moderate to high perifollicular staining for granzyme B in cytotoxic T cells, also associated with a poorer prognosis. The three tumor cell-related biomarkers were each associated with PFS following adjustment for FLIPI risk group and were present in greater than 10% of cases of a relatively large, previously untreated patient population receiving anti-CD20-based biologic therapy in a prospective, cooperative group setting.

This study has certain limitations. There were differences in terms of baseline characteristics between patients who were included in the TMA versus those not included, such that patients with biomarker data had higher-risk characteristics at baseline in terms of FLIPI risk group, disease state and presence of bulky disease. Follicular lymphoma is a heterogeneous disease with a wide range of treatment options spanning from observation to multi-agent chemotherapy regimens. Despite similar study entry criteria and chemotherapy-free treatment with anti-CD20-based therapy, outcomes differed for patients receiving lenalidomide and rituximab enrolled in CALGB 50803, who had a significantly superior PFS compared to patients enrolled in the other three trials (PFS HR=3.47, 95% CI: 2.11-5.72 [see Figure, Supplemental Digital Content 4). In addition, we observed a confounding interaction (e.g. Simpson's paradox) between interfollicular BCL6 staining and outcome in CALGB 50803, which precluded us from performing a typical univariate analysis of each marker in the pooled dataset of patients. We addressed this in our analytical approach by stratifying based on clinical trial and adjusting for FLIPI score in the in the primary analyses. For reference, unadjusted PFS curves for interfollicular BCL6 by trial are shown (Supplemental Digital Content 5) to demonstrate consistency across trials, supporting the validity of this approach.

High Ki67 proliferation index within follicles has been previously reported as an adverse prognostic factor in retrospective studies of non-uniformly treated follicular lymphoma patients with disease spanning all histologic grades and specifically in low-grade (grade 1-2 of 3) cases.[10, 32] Given that our study included patients with grade 3A disease and that the Ki67 proliferation index is known to increase with increasing histologic grade in follicular lymphoma based on number of centroblasts per high power field,[32] we also examined the association of this biomarker after excluding patients with grade 3A disease. Ki67 proliferation index within follicles remained significantly associated with PFS in patients with grade 1-2 cytology following adjustment for FLIPI and stratification by trial. These findings suggest that incorporation of Ki67 proliferation index into the routine diagnostic work-up of histologically low-grade follicular lymphoma may aid in prognostication, in conjunction with other clinical and biologic risk factors. As a nuclear stain, Ki67 is particularly well suited to automated analysis and was therefore the only stain that was evaluated quantitatively in our study using image analysis software. This not only strengthens the validity of the association with PFS, but also points to the utility of automated image analysis as a promising method to decrease interobserver variability, increase staining accuracy and report prognostic findings in a practical setting.[32]

The finding of interfollicular BCL6 positivity as an unfavorable prognostic feature in follicular lymphoma has not been previously identified to our knowledge. The similar but weaker association seen with PFS and CD10 compared to BCL6 supports the biologic validity of the observed association of interfollicular BCL6 positivity with inferior PFS, since both are germinal center B-cell associated markers. Moreover, combined interfollicular positivity for BCL6 and CD10, seen in 44/126 patients (35%), also showed a strong association with PFS, as well as a higher risk of early event by PFS24 (OR=9.07) compared with either marker alone (OR=5.25 for BCL6, OR=2.60 for CD10). The presence of neoplastic B cells (as identified by CD20 or other pan-B-cell antigen staining) in the interfollicular space is a recognized histopathologic feature of follicular lymphoma, but

interfollicular lymphoma cells often show absent, downregulated or more variable expression of germinal center markers compared to their expression in neoplastic follicles.[13, 33] BCL6 contributes to follicular lymphoma pathogenesis by repressing genes associated with exit from the germinal center and terminal B-cell differentiation, such as *PRDM1*,[34] thereby maintaining neoplastic cells at the germinal center stage of differentiation. Similarly, *EZH2*, a chromatin-modifying gene frequently mutated in follicular lymphoma, acts in concert with *BCL6* to silence *PRDM1* and other genes involved in terminal B-cell differentiation, including *IRF4* and *XBP1*, and to repress BCL6 target genes involved in cell cycle regulation, such as *CDKN1A*.[35] Expression of BCL6 in follicular lymphoma cells outside of the germinal center environment may thus result from mutations in *BCL6* itself or other chromatin-modifying genes involved in these pathways that allow tumor cells to escape the normal regulatory mechanisms of the B-cell follicle, reflecting a more aggressive disease phenotype. Further studies examining differences in the mutational profile of follicular lymphoma cases with and without interfollicular BCL6 expression may help to support this hypothesis.

Except for high perifollicular granzyme B expression, a feature seen in a relatively small number of cases (14/141, 10%), we did not find significant prognostic associations with biomarkers associated with microenvironmental immune cells, including PD1 and CD68. In addition, we found cases with >/=10% granzyme B-positive cells to have a shorter PFS, in contrast to the prior study demonstrating high perifollicular granzyme B expression in follicular lymphoma to be associated with longer PFS.[19] This difference may be related to multiple factors, including variation in the method used to score staining and treatment differences in the patient populations under study. In the prior study, the proportion of granzyme B-positive TILs was determined as a proportion of CD8-positive T cells, and both the proportion and intensity of granzyme B staining was used to determine an overall score. We opted to determine the proportion of granzyme B-positive cells among all cells without incorporating an assessment for staining intensity, a simpler approach that is more applicable to daily pathology practice. In addition, patients in the prior study were treated with rituximab in combination with chemotherapy rather than rituximab combined with other biologic agents. Similar factors may account for the lack of associations seen in this study compared to prior reports, particularly with regard to follicular PD1 staining of TILs[20, 36, 37] and density of CD68-positive tumor-associated macrophages.[14-18] However, early clinical trials showing activity of immune checkpoint inhibitors targeting macrophage pathways and the PD1/PDL1 axis point to a key role for these non-neoplastic cells in follicular lymphoma,[38-40] suggesting that further study of these biomarkers is warranted to assess their impact in risk stratification.

In summary, this study supports prior findings of the negative prognosis of high Ki67 within neoplastic follicles and identifies interfollicular BCL6 and CD10 expression in tumor cells as additional biologic risk factors in untreated follicular patients prospectively treated with rituximab or similar anti-CD20-based agents. Our findings support the prognostic utility of measuring Ki67 proliferation index in follicular lymphoma, in addition to determining the histologic grade based on centroblast count. These immunohistochemical stains represent promising biomarkers for risk stratification that warrant further validation in future trials of patients treated with various combinations of biologic agents. In addition, future studies of

differences in the cytogenetic and mutational profile between biomarker-positive and biomarker-negative cases may help to elucidate the molecular underpinnings of these risk factors and provide a basis for a more personalized therapeutic approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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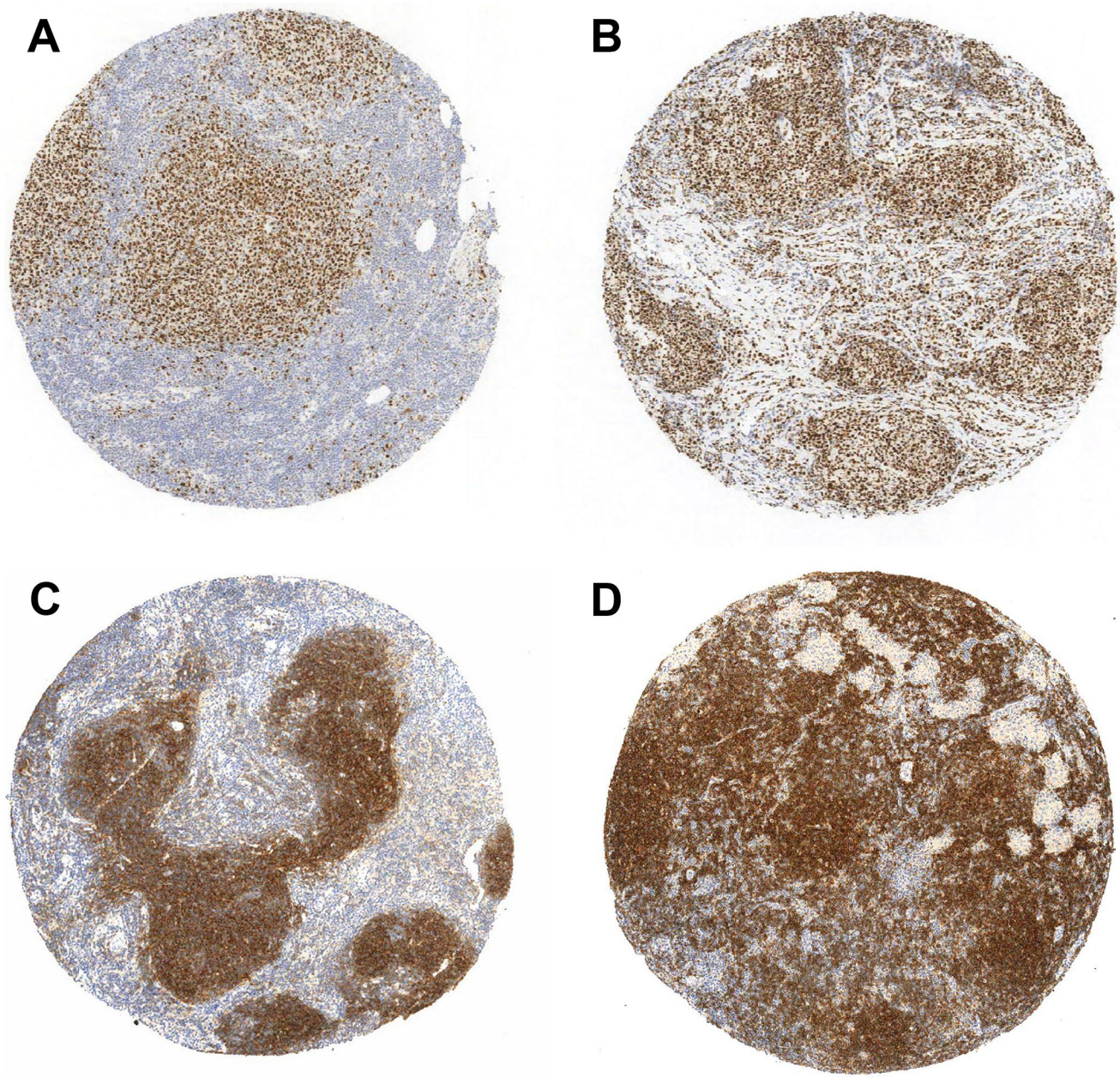

Figure 1: Representative histologic images of prognostic biomarkers in follicular lymphoma.
A-B. BCL6 staining restricted to neoplastic follicles (A, 40X) versus staining of both follicles and interfollicular space (B, 40X). C-D. CD10 staining restricted to neoplastic follicles (C, 40X) versus staining of both follicles and interfollicular areas (D, 40X).

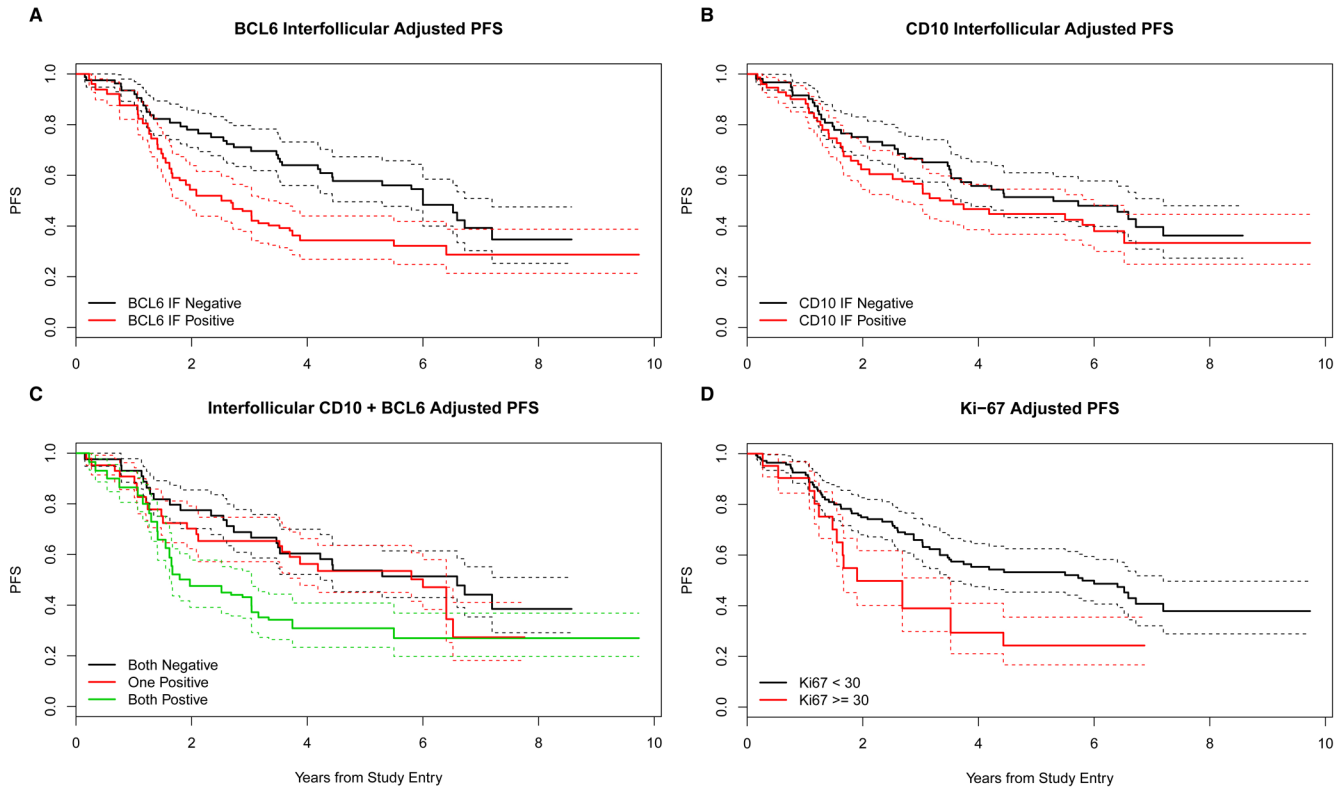

Figure 2: Adjusted progression-free survival (PFS) curves for prognostic biomarkers in follicular lymphoma.

A. Positive (red) vs. negative (black) staining for interfollicular BCL6. B. Positive (red) vs. negative (black) staining for interfollicular CD10. C. Concurrent interfollicular BCL6 and CD10 positivity (green) vs. one positive (red) or both negative (black). D. Ki67 proliferation index of >/=30% (red) vs. <30% (black) within neoplastic follicles. Dotted lines indicate 95% confidence intervals.

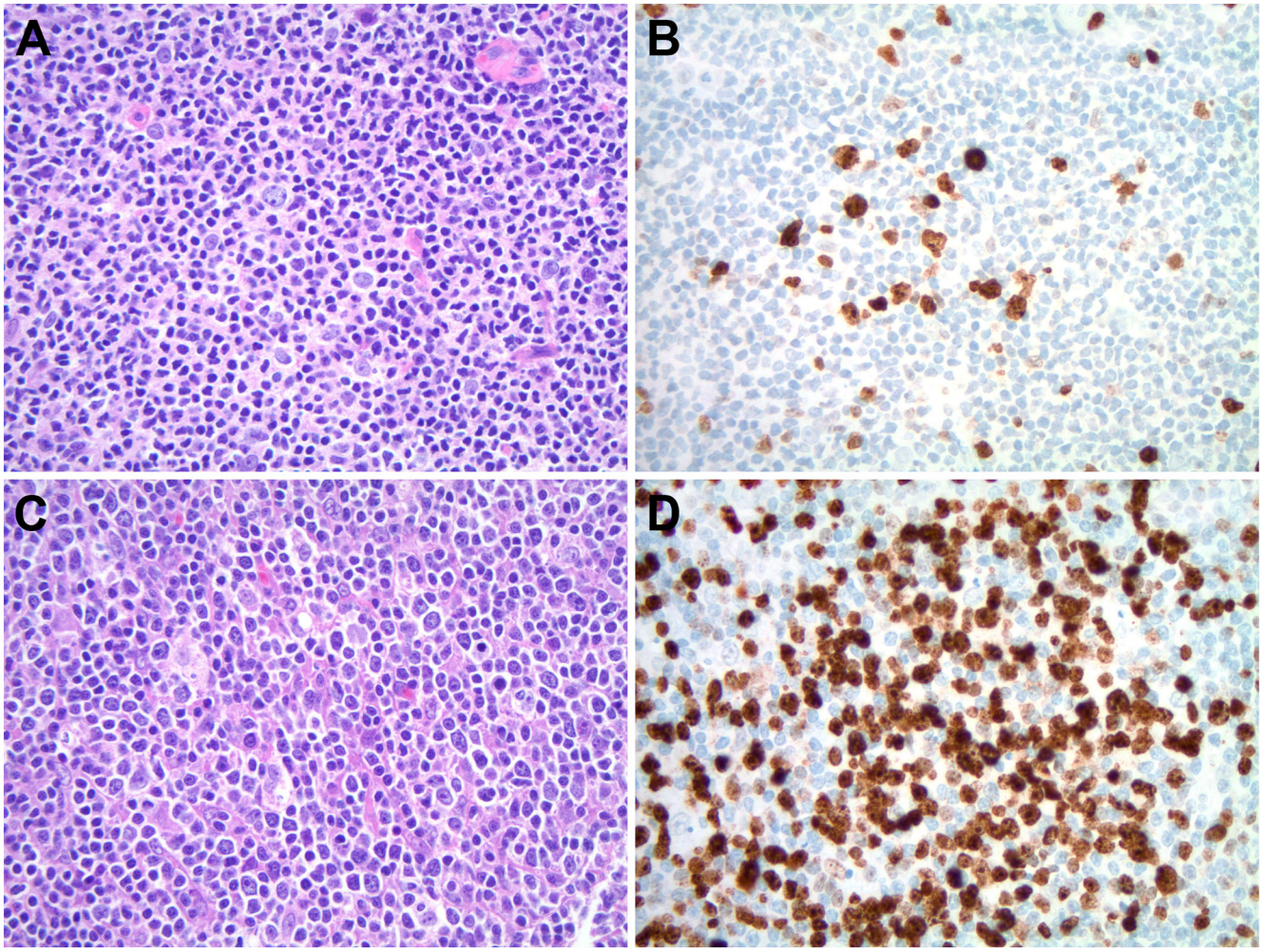

Figure 3: Ki67 proliferation index within neoplastic follicles.

A-B. A case of histologically low-grade (grade 1-2 of 3) follicular lymphoma with a low Ki67 proliferation index of 7% as determined by imaging analysis software (A, H&E, 40X; B, Ki67 stain, 40X). C-D. A separate case of histologically low-grade follicular lymphoma with a high Ki67 proliferation index of 49% (C, H&E, 40X; D, Ki67 stain, 40X).

Table 1:

Biomarkers Evaluated by Immunohistochemistry

Biomarker/ Stain	**Type (T/M)**	**Staining Characteristic Evaluated and Scoring Criteria***	**Basis for Scoring Cut-Off(s) Explored***
CD10	Germinal center (T)	Follicular and interfollicular staining (≥10% cells)	n/a
BCL6	Germinal center (T)	Follicular and interfollicular staining (≥10% cells)	n/a
MUM1 (IRF4)	Post-germinal center (T)	% cells positive (cut-off of 10%)	Kelley T, *et al. Leuk Lymphoma* 2007;48:2403-2411.
Blimp1	Post-germinal center (T)	Positive: >5% staining	n/a
FoxP1	Naïve B-cell (T)	% cells positive: cut-offs of <10%, 10-30%, 30-50%, >50%	Mottok A, *et al. Blood* 2018;131:226-235.
BCL2	Anti-apoptotic (T)	Positive (>/=20% staining) within follicles	n/a
Ki67 (MIB1)	Proliferation (T)	% cells positive within follicles (cut-off of >/=30%)	Wang SA, *et al. Am J Surg Pathol* 2005;29:1490-1496.
CD68	Macrophage (M)	# positive cells/hpf (cut-off of 16.8/hpf)	Richendollar BG, *et al. Hum Pathol* 2011;42:552-557.
PD1	T follicular helper (M)	% cells positive in follicles and interfollicular areas (cut-offs of </=5%, 6-33%, >33%)	Carreras J, *et al. J Clin Oncol* 2009;27:1470-1476.
FoxP3	T-regulatory (M)	Follicular, perifollicular or diffuse staining pattern	Farinha P, *et al. Blood* 2005;106:2169-74.
Granzyme B	Cytotoxic T-cell (M)	% cells positive in perifollicular areas (cut-offs of <10%, 10-30%, >30%)	Laurent C, *et al. Blood* 2011;118:5371-5379.

*Ki67 was scored by automated image analysis (Definiens, Carlsbad, CA). CD68 was scored per RIchendollar *et al* by each of three pathologists and average score computed. The remaining semiquantitative scores were the results of three independent reviews; disagreements (<5% of assessments) were resolved by consensus at a multiheaded microscope.

Abbreviations: T – tumor cell marker; M – microenvironmental marker; n/a – not applicable (exploratory staining patterns/cut-offs explored based on typical staining characteristics seen in practice); hpf – high power (40x objective lens) microscopic field.

Table 2:

Patient Characteristics

N	**154**
Age (years)	
Median (range)	55 (22-90)
Sex	
Male	81 (52.6%)
Female	73 (47.4%)
Missing	0
FLIPI	
Low 0-1	26 (17.1%)
Intermediate 2	95 (62.5%)
High 3-5	31 (20.4%)
Missing	2
Grade	
1-2	139 (93.3%)
3a	10 (6.7%)
Missing	5
Stage	
II (Bulky)	5 (3.3%)
III-V	148 (96.7%)
Missing	1
Bulky disease	
no	22 (17.1%)
yes	107 (82.9%)
Missing	25
LDH	
Normal	138 (89.6%)
Elevated	16 (10.4%)
ECOG PS	
0-1	148 (98.7%)
2	2 (1.3%)
Missing	4
Response	
No	23 (15.0%)
Yes	130 (85.0%)
Missing	1
PFS (years)	
Median (95% CI)	4.2 (3.3-6.4)

Abbreviations: ECOG: Eastern Cancer Oncology Group; FLIPI: follicular lymphoma international prognostic index; LDH: lactate dehydrogenase; PFS: progression-free survival; PS: performance status.

Table 3:

Results of Biomarker Analysis by Trial and Overall

Trial	50402	50701	50803	50901	Total
N	45	44	43	22	154
CD10 follicular					
Negative	5 (13.2%)	4 (10.8%)	3 (8.1%)	3 (15.8%)	15 (11.5%)
Positive	33 (86.8%)	33 (89.2%)	34 (91.9%)	16 (84.2%)	116 (88.5%)
Missing	7	7	6	3	23
CD10 interfollicular					
Negative	20 (50.0%)	22 (57.9%)	16 (43.2%)	9 (47.4%)	67 (50.0%)
Positive	20 (50.0%)	16 (42.1%)	21 (56.8%)	10 (52.6%)	67 (50.0%)
Missing	5	6	6	3	20
MUM1					
<10%	28 (65.1%)	35 (89.7%)	21 (56.8%)	10 (50.0%)	94 (67.6%)
>/=10%	15 (34.9%)	4 (10.3%)	16 (43.2%)	10 (50.0%)	45 (32.4%)
Missing	2	5	6	2	15
Granzyme B					
<10%	35 (85.4%)	40 (100.0%)	33 (82.5%)	19 (95.0%)	127 (90.1%)
10-30%	5 (12.2%)	0 (0.0%)	7 (17.5%)	1 (5.0%)	13 (9.2%)
>30%	1 (2.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.7%)
Missing	4	4	3	2	13
FoxP1					
<10%	17 (47.2%)	17 (58.6%)	18 (50.0%)	8 (40.0%)	60 (49.6%)
10-30%	3 (8.3%)	4 (13.8%)	1 (2.8%)	1 (5.0%)	9 (7.4%)
30-50%	2 (5.6%)	1 (3.4%)	3 (8.3%)	0 (0.0%)	6 (5.0%)
>50%	14 (38.9%)	7 (24.1%)	14 (38.9%)	11 (55.0%)	46 (38.0%)
Missing	9	15	7	2	33
FoxP3					
Diffuse	34 (82.9%)	31 (86.1%)	38 (95.0%)	14 (73.7%)	117 (86.0%)
Perifollicular	6 (14.6%)	3 (8.3%)	2 (5.0%)	3 (15.8%)	14 (10.3%)
Follicular	1 (2.4%)	2 (5.6%)	0 (0.0%)	2 (10.5%)	5 (3.7%)
Missing	4	8	3	3	18
BCL2					
Negative	2 (4.4%)	0 (0.0%)	3 (7.5%)	3 (14.3%)	8 (5.4%)
Positive	43 (95.6%)	42 (100.0%)	37 (92.5%)	18 (85.7%)	140 (94.6%)
Missing	0	2	3	1	6
Blimp1					
Negative	39 (100.0%)	39 (100.0%)	39 (100.0%)	20 (100.0%)	137 (100.0%)
Positive	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Missing	6	5	4	2	17

Trial	50402	50701	50803	50901	Total
BCL6 follicular					
Negative	5 (12.2%)	1 (2.9%)	0 (0.0%)	0 (0.0%)	6 (4.7%)
Positive	36 (87.8%)	33 (97.1%)	33 (100.0%)	20 (100.0%)	122 (95.3%)
Missing	4	10	10	2	26
BCL6 interfollicular					
Negative	26 (63.4%)	28 (71.8%)	4 (11.8%)	5 (25.0%)	63 (47.0%)
Positive	15 (36.6%)	11 (28.2%)	30 (88.2%)	15 (75.0%)	71 (53.0%)
Missing	4	5	9	2	20
CD68					
</= 16.8/hpf	30 (75.0%)	27 (73.0%)	15 (41.7%)	7 (35.0%)	79 (59.4%)
> 16.8/hpf	10 (25.0%)	10 (27.0%)	21 (58.3%)	13 (65.0%)	54 (40.6%)
Missing	5	7	7	2	21
PD1 follicular					
</=5%	11 (30.6%)	10 (33.3%)	12 (34.3%)	5 (26.3%)	38 (31.7%)
6-33%	23 (63.9%)	16 (53.3%)	16 (45.7%)	13 (68.4%)	68 (56.7%)
>33%	2 (5.6%)	4 (13.3%)	7 (20.0%)	1 (5.3%)	14 (11.7%)
Missing	9	14	8	3	34
PD1 interfollicular					
</=5%	31 (88.6%)	24 (80.0%)	21 (60.0%)	15 (78.9%)	91 (76.5%)
6-33%	3 (8.6%)	4 (13.3%)	13 (37.1%)	4 (21.1%)	24 (20.2%)
>33%	1 (2.9%)	2 (6.7%)	1 (2.9%)	0 (0.0%)	4 (3.4%)
Missing	10	14	8	3	35
Ki67 PI in follicles					
<30%	39 (95.1%)	34 (97.1%)	29 (87.9%)	10 (50.0%)	112 (86.8%)
>/=30%	2 (4.9%)	1 (2.9%)	4 (12.1%)	10 (50.0%)	17 (13.2%)
Missing	4	9	10	2	25
Progression Status					
Progression	30	31	10	18	89
No Progression	15	13	33	4	65

Abbreviations: hpf: high power (40x objective lens) microscopic field; PI: proliferation index.

Table 4:

Association of Biomarkers with Progression-Free Survival and PFS24

Type of analysis	Cox model for PFS			Logistic model for PFS24		
Marker (N)	**PFS HR**	**95% CI**	**p-value**	**PFS24 OR**	**95% CI**	**p-value**
CD10 follicular						
Negative (15)	ref			ref		
Positive (116)	1.82	0.83-4.00	0.13	2.74	0.66-11.42	0.16
CD10 interfollicular						
Negative (67)	ref			ref		
Positive (67)	1.48	0.94-2.34	0.09	2.60	1.05-6.43	0.03
MUM1						
<10% (94)	ref			ref		
>/=10% (45)	1.01	0.62-1.65	0.95	2.01	0.79-5.11	0.13
Granzyme B *						
<10% (127)	ref			ref		
>/=10% (14)	2.45	1.11-5.39	0.02	9.63	1.00-92.12	0.04
FoxP1 *						
<30% (69)	ref			ref		
>/=30 (52)	0.73	0.43-1.22	0.23	0.72	0.27-1.88	0.50
FoxP3						
Diffuse (117)	ref			ref		
Perifollicular (14)	1.01	0.48-2.14	0.97	1.32	0.37-4.71	0.66
Follicular (5)	2.06	0.78-5.47	0.14	3.73	0.37-36.93	0.25
BCL6 interfollicular						
Negative (63)	ref			ref		
Positive (71)	2.2	1.35-3.58	0.001	5.25	1.97-13.98	0.0009
CD68						
</= 16.8/hpf (79)	ref			ref		
> 16.8/hpf (54)	0.90	0.55-1.48	0.69	1.28	0.51-3.20	0.59
PD1 follicular *						
</=5% (38)	ref			ref		
>5% (82)	1.00	0.58-1.69	0.99	1.42	0.49-4.07	0.51
PD1 interfollicular *						
</=5% (91)	ref			ref		
>5% (28)	0.79	0.41-1.50	0.47	1.30	0.39-4.29	0.65
Ki67 PI in follicles						
<30% (112)	ref			ref		
>/=30% (17)	2.47	1.33-4.61	0.004	7.49	1.77-31.73	0.006

Cox model stratified on study (50803 vs other) and adjusted for FLIPI group. Logistic regression model adjusted for study (50803 vs. other) and FLIPI group.

*Certain semi-quantitative subgroups containing few cases combined (granzyme B 10-30% and >30%, FOXP1 <10% and 10-30%, FOXP1 30-50% and >50%, PD1 follicular 6-33% and >33%, and PD1 interfollicular 6-33% and >33%). Blimp1, BCL2 and BCL6 follicular not listed because all or nearly all (>90%) cases were negative for the biomarker.

Abbreviations: CI: confidence interval; FLIPI: follicular lymphoma international prognostic index; hpf: high power (40x objective lens) microscopic field; HR: hazard ratio; OR: odds ratio; PFS: progression-free survival; PFS24: progression free survival at 24 months PI: proliferation index; ref: reference.