Similarities of prosurvival signals in Bcl-2-positive and Bcl-2-negative follicular lymphomas identified by reverse phase protein microarray

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Overexpression of Bcl-2 protein has been known to play a role in the pathogenesis of follicular lymphoma (FL). However, 10–15% of FLs are negative for Bcl-2 by immunohistochemistry, raising the possibility that another gene product(s) may provide prosurvival signal(s). We used reverse phase protein microarray to analyze lysates of follicle center cells isolated by laser capture microdissection from: Bcl-2+ FL, Bcl-2– FL and reactive follicular hyperplasia (FH) (nine cases each group). TUNEL assay confirmed similar and reduced levels of apoptosis in Bcl-2+ FL and Bcl-2– FL, indicating the likelihood of Bcl-2-independent inhibition of apoptosis. Arrays were quantitatively analyzed with antibodies to proteins involved in the apoptotic pathway. As expected, Bcl-2 levels were up to eight-fold higher in Bcl-2+ FL than in FH and Bcl-2– FL. However, there was no difference in levels of Mcl-1 and survivin among these three groups. Bcl-X_L showed a trend for increased expression in Bcl-2– FL as compared with Bcl-2+ FL, although the differences did not reach statistical significance ($P>0.1$). The increase in Bcl-X_L may provide an alternative antiapoptotic signal in FL negative for Bcl-2 protein. Interestingly, Bax expression was higher in FL (Bcl-2+ or –) than in FH ($P=0.001$). Notably, phospho-Akt (Ser-473) was increased in FL (Bcl-2+ or –) ($P<0.03$) with increased phospho-Bad (Ser-136), as compared with levels in FH. The activation of the Akt/Bad pathway provides further evidence of prosurvival signals in FL, independent of Bcl-2 alone. These data suggest that nodal FL represents a single disease with a final common biochemical pathway.

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Follicular lymphoma (FL) is one of the most common types of lymphoma in Western countries. It is characterized by a clinically indolent course, but is incurable in advanced stages by conventional treatment. The majority of FLs overexpress Bcl-2 protein as a consequence of the t(14;18), found in approximately 85–90% of cases. The *BCL-2* gene was first discovered in FL, and has been considered the critical oncogene involved in pathogenesis of this tumor.^{1–4} Expression of the Bcl-2 protein results in the inhibition of apoptosis, thought to be a critical pathogenetic event in the development of FL.^{5–7}

However, approximately 10–15% of cases of FL are negative for Bcl-2 protein,^{8,9} suggesting the possibility that another gene product(s) may provide a prosurvival signal(s). Furthermore, B cells carrying the t(14;18) can be identified in the peripheral blood and lymphoid tissues of healthy individuals^{10–12} and transgenic mice bearing the Bcl-2/IgH translocation developed polyclonal B-cell hyperplasia but not a monoclonal lymphoproliferative disorder.^{7,13} These findings suggest that Bcl-2 alone may not be sufficient to cause FL, and that other genetic changes are also involved in this neoplastic process.

Bcl-2 protein and other protein products involved in the apoptosis pathway have been studied by immunohistochemistry.^{14–16} Although immunohistochemistry can provide clues to the abundance of cellular antigens directly on tissue sections, it is not quantitative, and it is difficult to examine multiple

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proteins simultaneously within an individual cell type. In addition, the presence of heterogeneous cellular populations can make interpretation difficult.

Laser capture microdissection is a recently developed technique that enables the reliable procurement of specific cell types from a tissue section under direct microscopic visualization for a highly enriched starting source of cellular material. RNA can be isolated from the microdissected cells and subjected to cDNA microarray analysis.¹⁷ Both FL and normal germinal center B cells have been studied using cDNA arrays.^{18,19} However, mRNA expression may not correlate directly with protein levels, and RNA expression does not provide information on post-translation modifications, such as phosphorylation, glycosylation or cleavage, which may be essential for protein function.²⁰

Recently, a reverse phase protein microarray technique has been developed, which, in contrast to previous antibody ligand or heterogeneous tissue fragment arrays, immobilizes whole protein lysates procured by Laser Capture Microdissection from biologically relevant populations.^{20,21} This technique combines a high degree of sensitivity and linearity, making it possible to study in detail components of the prosurvival or antiapoptotic pathway.²¹ We utilized this technique to study FL cells to examine the relevant roles of Bcl-2 and other molecules in the pathogenesis of FL. In particular, we wished to investigate cases of FL that were negative for Bcl-2 protein, to identify molecules that might substitute for Bcl-2 in inhibition of apoptosis. We also sought to compare cases of FL positive and negative for Bcl-2 protein, to determine if they involved a biochemically similar pathway. We sought to determine if Bcl-2-positive and -negative FLs are part of a single disease entity, utilizing different molecules to arrive at a common functional state or different diseases that share only morphological similarity. Cells isolated from FL were compared with B cells isolated from normal germinal centers in order to study the prosurvival pathway in B cells derived from common cellular and functional origins.

Materials and methods

Case Selection

The surgical pathology reports of 180 biopsy specimens diagnosed as FL at the Warren Grant Magnuson Clinical Center from 1994 to 2001 were reviewed. All patients were being followed on a treatment protocol approved by the Institutional Review Board of the National Cancer Institute. A portion of each biopsy specimen had been cryopreserved in an Optimal Cutting Temperature (OCT) embedding compound (Sakura Tissue Tek; Torrance, CA, USA) at the time of diagnosis, and stored over liquid nitrogen. Immunohistochemical studies for

Bcl-2 protein had been performed at diagnosis on paraffin sections using previously published techniques.^{22,23} Cases negative for Bcl-2 in paraffin sections were confirmed as negative in frozen sections. Nine cases were identified as negative for Bcl-2 protein. For comparison, we selected nine cases of FL positive for Bcl-2 protein, as well as nine cases diagnosed as reactive FH in which cryopreserved tissue was available. The FL cases were graded according to the World Health Organization (WHO) classification.²⁴ All cases were Grade I or II, with one case of Grade IIIa, Bcl-2 positive, and one case of Grade IIIa, Bcl-2 negative. All cases of FL (Bcl-2 protein positive and negative) were studied for the *JH/BCL2* rearrangement by polymerase chain reaction (PCR) using previously published techniques (see below).²³

Laser Capture Microdissection (LCM) and Protein Microarray Preparation

Microdissection was performed on cryosections using a Pixcell 200 Laser Capture Microdissection System (Arcturus Engineering, Mountain View, CA, USA), as described before.²¹ Briefly, the frozen tissue sections were stained with a modified hematoxylin and eosin staining protocol and treated sequentially in 100, 95 and 70% ethanols, HPLC grade water, hematoxylin (Sigma, St Louis, MO, USA), HPLC grade water, bluing solution (Sigma, St Louis, MO, USA), 70, 95 and 100% ethanol for 20 s each with a final dehydration in xylene. All staining baths contain 10 mmol complete[™] (Boehringer Mannheim, Germany) protease inhibitors. 6000-LCM shots were acquired per case, targeting either neoplastic follicles (FL cases), or reactive follicles (FH cases), respectively. For each case, the sampled cells were taken from 8 to 10 randomly selected follicles, with no attempt to select centrocytes or centroblasts.

The preparation of protein microarrays and analysis of data have been previously described in detail.²⁵ Briefly, the microdissected cells were lysed in 40 μ l of lysing buffer containing 1:1 mixture of 2 $\times$ SDS electrophoresis buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 2% β -mercaptoethanol) and Tissue Protein Extraction Reagent (TPER, Pierce, Rockford, IL, USA) and incubated for 2 h at 65°C. After cell lysis, samples were boiled between 3 and 5 min each and 2 nl of the lysate was arrayed with a pin and ring GMSE 470 microarrayer (Affymetrix) using a 375- μ m pin onto nitrocellulose slides with a glass backing (Schleicher and Schuell, Keene, NH, USA). Two-fold dilutions were made manually using lysing buffer in a 96-well plate. Each dot and its successive dilutions represent the lysate from all cells dissected from a single case (6000 LCM shots).

Staining was carried out on an automated slide stainer (DAKO, Carpinteria, CA, USA) using the

catalyzed signal amplification system as per the manufacturer's recommendation. Briefly, after micro-application of cellular lysates, the slides were treated for 15 min with Reblot™ (Chemicon, Temecula, CA, USA) and subsequently washed three times, 10 min each, with TBS washing buffer (300 mmol NaCl, 0.1% Tween-20, 50 mmol Tris, pH 7.6). After treatment, the arrays were blocked in a 0.5% casein solution for 30 min under constant rocking. Between each of the following steps, arrayed slides were washed three times 5 min each with TBS washing buffer. Endogenous biotin was blocked using the biotin blocking kit (DAKO, Carpinteria, CA, USA) for 5 min followed by the application of protein block (DAKO, Carpinteria, CA, USA) for 5 min, primary antibody diluted in antibody diluent (DAKO, Carpinteria, CA, USA) at various concentrations for 30 min and finally second link antibody (DAKO, Carpinteria, CA, USA) at a concentration of 1:100 for anti-mouse and neat for anti-rabbit for 30 min. Proper secondary antibody alone controls were included. Primary antibodies with dilutions are as follows: Bcl-2 (DAKO, Carpinteria, CA), 1:50; Mcl-1 (Novocastra, Newcastle upon Tyne, UK), 1:50; Bcl-X_L, 1:1000 (Cell Signaling Technology, Beverly, MA, USA); Bax (BD Pharmingen, La Jolla, CA, USA), 1:3000; survivin (Novus, Littleton, CO, USA), 1:1000; phospho-Akt (S473) and Akt (Cell Signaling, Beverly, MA, USA), 1:100; phospho-Bad (S136) and Bad (Cell Signaling), 1:100; MIB-1 (DAKO), 1:50; and actin (Zymed, San Francisco, CA, USA), 1:1000. The specificity of each antibody had been confirmed by Western blot.²¹

Amplification and staining were carried out as follows: the microarray was subsequently treated with a streptavidin–biotin complex (DAKO, Carpinteria, CA, USA) solution for 15 min, amplification reagent (biotinyl tyramide and hydrogen peroxidase) for 15 min, streptavidin-peroxidase for 15 min, and finally stained using 3,3'-diaminobenzidine tetrahydrochloride as chromogen.

Image and Statistical Analyses

Stained arrays were scanned on a UMAX scanner with Adobe PhotoShop 5.5 at a resolution of 600 dpi for analysis. Scanned images (saved as a tif. file) were analyzed with Image Quant (Molecular Dynamics, Sunnyvale, CA, USA). The Student's *t*-test (unpaired, two-tailed) was used to compare group differences in normalized protein expression levels against actin as a standard. The vertical axes of Figures 1–6 represent the normalization ratios. Graphs show mean $\pm$ s.e., with the s.e. bars reflecting the variability among the sampled cases, not dot replicates. Prior studies have shown a good correlation over the dilution curve, with a correlation coefficient r^2 of 0.973.²¹

Immunohistochemistry

Corresponding available paraffin-embedded tissue blocks were retrieved and sectioned. Immunohistochemical studies were performed with primary antibodies, using an automated immunostainer (Ventana Medical System, Tucson, AZ, USA) according to the manufacturer's protocols with minor modifications, as described previously.²³ Antigen retrieval was performed using a Tender Cooler (Nordicware, Minneapolis, MN, USA) with citrate buffer, as described previously.²² Primary antibodies with dilutions used in the panel for FL diagnosis are as follows: CD20 (Dako, Carpinteria, CA, USA), 1:200; CD3 (Dako), 1:100; Bcl-2 (Dako), 1:20; CD10 (Novocastra, Newcastle Upon Tyne, UK), 1:40; and Bcl-6 (Dako), 1:20. All cases were also stained with an antibody to Bax (BD Pharmingen) at a dilution of 1:2000.

Detection of BCL2/IgH Gene Rearrangement by PCR

The *BCL2*/immunoglobulin (Ig) heavy-chain gene translocation was analyzed by PCR on paraffin-embedded or frozen tissue, as described before.²³ Published primers for *BCL2* and *JH* were used for the detection of t(14;18) translocation involving the major breakpoint region (MBR).²⁶ The specificity of the *BCL2* amplification product was confirmed by Southern blotting and hybridization with a digoxigenin (DIG)-labeled oligonucleotide. Bound probe was detected with an alkaline phosphatase-labeled anti-DIG Fab fragment and CSPD as chemiluminescence substrate (Boehringer Mannheim, Indianapolis, IN, USA). In all experiments, test samples were run in parallel with positive and negative controls. Approximately 50–60% of breakpoints in translocation-positive FL cluster in the MBR, with an overall detection rate in our laboratory and the literature of 50%.^{27,28}

TUNEL Assay

Apoptotic cells were stained using the terminal deoxynucleotidyl transferase TdT-FragEL DNA Fragmentation Detection kit (Oncogene Research Products, San Diego, USA) according to the manufacturer's protocol. Briefly, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in descending grades (100–70%) of ethanol. The sections were rinsed with tri-buffered saline (TBS, pH 7.6) and incubated with proteinase K (20 μ g/ml in 10 mM Tris pH 8.0) for 20 min at room temperature. The sections were rinsed with TBS and incubated with 3% H₂O₂ in methanol for 5 min at room temperature to block endogenous peroxidase activity. The sections were rinsed with TBS and incubated with TdT Equilibration Buffer for 30 min at room temperature. After carefully blotting the TdT Equilibration Buffer, TdT Labeling Reaction

Mixture was immediately applied to the sections, which were subsequently incubated with coverslips in a humidified chamber at 37°C for 1.5 h. The sections were rinsed with TBS and incubated with Stop Solution for 5 min to terminate the labeling reaction. The sections were rinsed with TBS and incubated with Blocking Buffer for 30 min at room temperature. Finally, the sections were rinsed with TBS and incubated with a diaminobenzidine chromogen solution for 10 min at room temperature and counterstained with methyl green. The sections were dehydrated and mounted under glass coverslips. Positive and negative controls were included. Apoptotic cells were counted in five high power field ($\times 40$) of reactive or neoplastic follicles, which was expressed as apoptotic index (AI, apoptotic cells/mm²), as described previously.^{16,29}

Results

Histologic and Genotypic Characterization

All cases were reviewed and showed the characteristic histological and immunophenotypic features for a diagnosis of FL or reactive FH, respectively.²⁴ (Figure 1a). While reactive and neoplastic follicles were uniformly positive for the B-cell marker CD20, a mixture of scattered CD3-positive T cells was also noted. The extent of admixed T cells was scored from 1+ (scattered, ~ 250 /hpf) to 3+ (numerous, ~ 950 /hpf). T-cell numbers were similar in FH- and Bcl-2-negative FL, although T cells in Bcl-2-positive FL were mildly decreased (Table 1). Staining for Bcl-2 protein was correlated with genetic analysis for the t(14;18) by PCR.

Expression of Bcl-2 Family Proteins

The microarray stained for Bcl-2 protein is shown in Figure 1b, with quantitative data in Figure 1c. Bcl-2 protein was approximately eight-fold higher in Bcl-2-positive FL than in reactive follicular hyperplasia. However, Bcl-2-negative FL, despite negative staining by immunohistochemistry, revealed weak but statistically significant Bcl-2 protein expression by microarray when compared to FH ($P < 0.008$).

Mcl-1 is one of the major antiapoptotic proteins in the Bcl-2 family. A reciprocal temporal relationship of Mcl-1 and Bcl-2 has been reported in some lymphoid tissues or cell lines.^{15,30} However, there were no significant differences observed in Mcl-1 expression among the three groups of cases ($P > 0.3$) (Figure 2).

Bcl-X_L is an antiapoptotic member of the Bcl-2 family. Bcl-X_L has been shown to be expressed in both FL cells and germinal center cells, and its downregulation leads to apoptosis.³¹ Interestingly, Bcl-X_L was expressed at higher levels in Bcl-2-negative FL than in Bcl-2-positive FL, although these differences did not reach statistical signifi-

Figure 1 Levels of Bcl-2 protein in Bcl-2-positive FL, Bcl-2-negative FL and FH. (a) Immunohistochemical staining for Bcl-2 protein (bottom row) vs H and E staining (top row) of paraffin-embedded tissue sections. One representative case is shown from each study group. (b) Protein microarray stained with anti-Bcl-2. a = FH, b = Bcl-2-positive FL, c = Bcl-2-negative FL. Three cases are shown from each study group with spots representing two-fold serial dilutions made from the left to right. (c) Normalized levels of Bcl-2 protein in FH, Bcl-2-positive FL and Bcl-2-negative FL. The data were obtained by scanning stained arrays followed by analysis with Image Quant. Nine cases were analyzed in each group. (Figures 2–7 represent data displayed in a similar manner).

cance ($P > 0.1$) (Figure 3). There was no statistical difference in the expression of Bcl-X_L vs FL, either Bcl-2 positive or negative.

Bax is a prototype of the proapoptotic proteins in the Bcl-2 family, and the ratio of Bcl-2 to Bax protein determines cell survival or death. Therefore, low levels of Bax could lead to a prosurvival signal. Unexpectedly, a significant increase in Bax protein was seen in both Bcl-2-positive and Bcl-2-negative FL when compared to the Bax level in FH ($P = 0.001$) (Figure 4a). We pursued this observation by immunohistochemical staining for Bax protein on paraffin-embedded tissue sections. The results were consistent with the microarray finding. Reactive follicles showed overall weak staining with scattered strongly positive cells, while neoplastic follicles (Bcl-2 positive or negative) revealed a uniformly strong signal (Figure 4b and Table 1).

Expression of Survivin

Survivin is a member of inhibitors of apoptosis (IAPs) and has been reported to be increased in a variety of tumors.³² However, levels of survivin

Table 1 Characterization of cases studied by reverse phase microarray

Age/sex (years)	Grade (WHO)	BCL2/IgH (PCR)	CD3 (follicles)	Bax (follicles)
<i>Follicular lymphoma, Bcl-2 positive</i>				
34/M	I	+	1+	4+
57/F	I	+	3+	4+
54/F	II	+	1+	3+
52/M	II	+	1+	4+
24/M	II	+	1+	4+
47/F	IIIa	+	1+	3+
58/F	II	+	2+	4+
37/F	I	+	1+	3+
32/M	II	+	2+	ND
<i>Follicular lymphoma, Bcl-2 negative</i>				
47/M	II	+ ^a	2+	4+
48/F	II	—	2+	3+
41/F	I	+	Un	4+
70/F	II	—	2+	3+
44/M	I	—	1+	3+
75/F	IIIa	—	2+	2+
55/M	II	+	3+	3+
73/F	I	Un	2+	4+
80/F	II	—	1+	Un
<i>Reactive follicular hyperplasia</i>				
18/F			2+	1+
22/M			ND	ND
20/F			2+	1+
29/M			2+	3+
52/M			1+	1+
25/F			3+	1+
86/M			2+	2+
48/F			2+	2+
48/M			2+	1+

CD3: 1+, scattered positive cells; 2+, frequent positive cells; 3+, numerous positive cells (see results).

Bax: 1+, very weakly positive; 2+, weakly positive; 3+, positive; 4+, strongly positive.

Un: Technically unsatisfactory.

ND: Not done.

^aDetected by FISH.

Figure 2 Mcl-1 expression analyzed by reverse phase protein microarray in Bcl-2-positive FL, Bcl-2-negative FL and FH. No statistical difference was present in the levels of Mcl-1 among the three groups of cases.

appeared decreased rather than increased in Bcl-2-positive and Bcl-2-negative FL when compared to levels in FH, although this change was not statistically significant ($P > 0.07$ and > 0.1) (Figure 5).

Figure 3 Expression of BCL-XL in Bcl-2-positive FL, Bcl-2-negative FL and FH. A trend towards increased expression of BCL-XL is noted in Bcl-2-negative FL in comparison with Bcl-2-positive FL ($P > 0.1$). There were no significant differences in expression between FH and FL, Bcl-2 positive or negative.

Figure 4 Expression of Bax in Bcl-2-positive FL, Bcl-2-negative FL and FH. (a) A significant increase in Bax expression was noted by reverse phase protein microarray in Bcl-2-positive FL and Bcl-2-negative FL as compared with the levels in FH (nine cases each group). (b) Immunohistochemical stains for Bax protein (bottom) on paraffin-embedded tissue sections were consistent with the microarray result (one representative case shown from each group). The top panel represents H and E-stained sections from the corresponding case.

Expression of Akt and Bad

Akt is a signal transduction kinase involved in growth factor-induced survival. It has been demonstrated to inhibit apoptosis and play an important role in tumorigenesis.^{21,33–36} In the present study, phosphorylated Akt (Ser-473) was significantly increased in both Bcl-2-positive and Bcl-2-negative FL ($P < 0.006$ and < 0.03) (Figure 6a), while total Akt was also correspondingly increased ($P < 0.03$, data not shown). Immunohistochemical studies of Akt/p-Akt were not performed. Phospho-Akt is a low abundance protein that is very difficult to quantify by immunohistochemistry. Moreover, variations in staining intensity are highly affected by tissue preparation and antigen retrieval techniques.

Figure 5 Expression of survivin analyzed by reverse phase protein microarray. Relatively decreased levels of survivin were noted in FL (Bcl-2 positive or negative) as compared to FH, but no statistical difference was found.

Figure 6 Activation of Akt/Bad pathway in FL. Levels of phospho-Akt (a) and phospho-Bad (b) were analyzed by reverse phase protein microarray. Phospho-Akt (Ser-473) levels were significantly higher in FL (Bcl-2 positive or negative) than in FH. Phospho-Bad (Ser-136) was significantly increased in Bcl-2-negative FL. An increase was also noted in Bcl-2-positive FL, although it did not reach statistically significant levels.

As the increase in expression levels was in the range of 50%, immunohistochemical studies were believed not likely to be informative as to levels of expression.

One of most important mechanisms by which activated Akt plays a prosurvival role is to phosphorylate Bad and inactivate its activity as a proapoptotic protein in the Bcl-2 family. In the present study, levels of phosphorylated Bad (Ser-136) were significantly increased in Bcl-2-negative FL ($P < 0.05$) (Figure 6b). Phospho-Bad also appeared increased in Bcl-2-positive FL, although the change was not statistically significant ($P = 0.08$).

Apoptotic Index and Proliferative Rate

We questioned whether Bcl-2-negative FLs would demonstrate comparable inhibition of apoptosis to

Figure 7 Apoptotic activity and proliferative rate in Bcl-2-positive FL, Bcl-2-negative FL and FH. A marked decrease in apoptotic activity was seen by TUNEL assay on paraffin-embedded tissue sections (a), with a similar extent of apoptosis inhibition as shown by AI (apoptotic cells/mm²) (b), in Bcl-2-positive FL and Bcl-2-negative FL. (c) The proliferative rate estimated by anti-MIB1 on the microarray was correlated with the AI.

Bcl-2-positive FL. Apoptotic activity was analyzed by TUNEL assay and expressed as the AI (apoptotic cells/mm²). When compared to FH, FL showed a marked decrease in apoptotic activity ($P < 0.0006$). However, there was no difference in the AI between Bcl-2-positive and Bcl-2-negative FL ($P > 0.7$) (Figure 7a and b). The proliferative rate shown by Ki-67/MIB1 on reverse phase protein microarray was concordant with the AI (Figure 7c), and was slightly higher in Bcl-2-negative FL than in Bcl-2-positive FL, although these differences were not significant.

Discussion

FL is considered the neoplastic counterpart of the normal germinal center.³⁷⁻³⁹ Upon exposure to antigen, follicle center B cells proliferate and their Ig heavy-chain (IgH) genes undergo somatic mutation with positive and negative selection. Meanwhile, expression of Bcl-2 is switched off. Those B cells with an unfavorable mutation status, binding antigen with only low-affinity Ig, are eliminated by apoptosis. In contrast, B cells with favorable

mutations resulting in high-affinity Ig receive survival signals allowing them to differentiate further, and leave the germinal center. In FL, follicle center cells bear the t(14;18) that creates a derivative chromosome 14 on which the BCL2 gene is juxtaposed to IgH sequences.¹⁻⁵ This leads to the deregulation of the BCL2 gene with constitutive overexpression of Bcl-2 protein and inhibition of apoptosis normally occurring in germinal centers.

The expression of Bcl-2 protein in FL B cells is a hallmark of the disease, and immunohistochemical staining for Bcl-2 protein has been widely used to differentiate FL from reactive follicular hyperplasia. However, it has been noted that approximately 10–15% of FLs are immunohistochemically negative for Bcl-2 protein.^{8,9,40} Prior studies have investigated other prosurvival signals that might be present in FL, and increased levels of some proteins such as Bcl-X_L have been identified.³¹ Thus, the inhibition of apoptosis in FL is unlikely to be solely dependent on Bcl-2.

However, comparisons between Bcl-2-positive and Bcl-2-negative FL have not been performed, and it is unknown if there are prosurvival signals unique to Bcl-2-negative FL. In addition, we questioned whether all FLs exhibited disruption of a common biochemical pathway, and if other constituents of the apoptotic pathway would be expressed at similar or different levels in these two phenotypic forms of the disease. Differences might suggest that Bcl-2-positive and -negative FLs were in fact morphologically similar, but biologically distinct disease entities.⁴¹ We used reverse phase protein microarrays in conjunction with laser capture microdissection to measure quantitatively alterations of key apoptotic proteins in neoplastic and normal follicle center B- cells.²¹

We used this type of protein microarray because of its ability to generate a direct comparison of analytes from microscopic quantities of cells directly from tissue specimens. Laser capture microdissection provided the starting point material so that tissue heterogeneity issues could be minimized, and analysis of the prosurvival and apoptosis signaling pathways could be directly queried for a distinct cell population. As each patient sample is arrayed in a miniature dilution curve, we can analyze any given analyte in the linear dynamic range of the specific antibody-analyte concentration. Since all samples are arrayed on the same slide, a direct comparison between analyte concentrations from one specimen to the next can be made with very high precision.²⁰

Somewhat unexpectedly, we found that most proapoptotic and antiapoptotic proteins were expressed at similar levels in Bcl-2-positive and Bcl-2-negative FL. Studies of the apoptotic index also showed similarities between Bcl-2-positive and Bcl-2-negative cases. These results suggest that a common disruption of the apoptotic pathway underlies all cases of FL, regardless of the expression of Bcl-2 protein. For example, Mcl-1 is a major

antiapoptotic protein and Mcl-1 has been found to be overexpressed in some lymphoid neoplasms, in particular, multiple myeloma.^{42,43} We questioned as to whether Mcl-1 could substitute for Bcl-2 in Bcl-2-negative FL. However, we failed to demonstrate a significant difference in Mcl-1 levels among Bcl-2-positive FL, Bcl-2-negative FL and FH.

Prior studies had suggested a potential role for Bcl-X_L in FL, identifying it as one of the antiapoptotic proteins expressed along with Bcl-2 and Mcl-1. Bcl-X_L is expressed in both normal germinal center cells and FL cells, and the downregulation of Bcl-X_L is associated with rapid onset of apoptosis in both cell types.³¹ However, activation of FL cells *in vitro* via CD40 leads to maintenance or even increased expression of Bcl-X_L, producing an antiapoptotic signal. The fact that CD40 ligand-positive T cells are found within the neoplastic follicles of FL further suggested that this mechanism might operate *in vivo* to increase levels of Bcl-X_L.³¹ However, prior studies did not examine the levels of expression of Bcl-X_L in relation to other antiapoptotic molecules such as Bcl-2 or Mcl-1. We found higher levels of Bcl-X_L in Bcl-2-negative FL than in Bcl-2-positive FL, although these differences did not reach statistical significance ($P > 0.1$). However, our results do suggest that Bcl-X_L is certainly expressed and may be especially important in providing a prosurvival signal in cases of FL negative for Bcl-2 protein.

Bcl-2 family members are classified into two groups: antiapoptotic and proapoptotic proteins, and studies have shown that it is the ratio of the two that determines cell survival or death.⁴⁴ Therefore, a decrease in one or more of the proapoptotic proteins in Bcl-2-negative FL could overcome an absence of Bcl-2. Bax is one of major proapoptotic proteins, and an earlier study had identified expression in most B-cell lymphomas, regardless of histological grade.⁴⁵ Interestingly, Bax was significantly increased, and to a similar extent, in both Bcl-2-positive and Bcl-2-negative FL, in comparison with FH. Increased levels of expression were confirmed by immunohistochemical studies. The significance of this finding is unknown, but Bax may be increased to 'compensate for' decreased levels of other proapoptotic proteins, or to 'pair with' one or more of enhanced antiapoptotic signals. The detected Bax may also represent mutated Bax protein with aberrant function, as Bax gene mutations have been reported in some cancers.⁴⁶⁻⁴⁸

We also examined the prosurvival protein survivin, a recently characterized mammalian member of the IAP family of proteins. Its overexpression in cancers has suggested a general role of apoptosis inhibition in tumor progression. In addition, survivin is required for proper execution of mitosis and cell division. Its expression is cell cycle dependent, undetectable in quiescent tissues and abundant in proliferating cells (up to 40-fold RNA levels in G2/M).⁴⁹ In the present study, survivin appeared decreased, rather than increased in FL, when

compared to the levels in FH. This finding is most likely due to the marked increased expression of survivin associated with rapid proliferation within reactive germinal centers.

Recently, the serine/threonine protein kinase, Akt or protein kinase B, has emerged as one of the crucial regulators of apoptosis and has been increasingly linked to cell survival and tumorigenesis.^{21,33–36,50} Akt is activated downstream of phosphatidylinositol-3 kinase (PI-3 kinase) by various growth and survival factors. One of the actions of activated Akt includes phosphorylation of proteins involved in the apoptotic cascade and cell survival. The Bcl-2 family member Bad, a proapoptotic protein, is a target of activated Akt.^{51,52} The phosphorylation of Bad inactivates its activity by creating a binding site for a 14-3-3-adaptor protein that retains Bad in the cytosol, and prevents its dimerization with Bcl-XL.⁵³ The Akt kinase has been shown to be activated in multiple myeloma cells,⁵⁴ but studies of the PI-3-kinase/Akt pathway in B-cell lymphomas have been limited to cell lines.⁵⁵

In the present study, phosphorylated Akt was significantly increased in both Bcl-2-positive and Bcl-2-negative FL when compared to baseline levels in FH. Phosphorylated Bad was correspondingly significantly increased in Bcl-2-negative FL, although the increase in Bcl-2-positive FL did not reach statistically significant levels. While the expression of total Akt was concurrently increased in FL, as has been seen in other neoplasms,³⁶ the levels of total Bad protein did not change significantly (data not shown). These findings suggest that the Akt/Bad pathway may play an important role in the suppression of apoptosis in FL, particularly in Bcl-2-negative FL. In addition to the phosphorylation of Bad, Akt has been reported to inhibit apoptosis by upregulating the levels of Mcl-1, another antiapoptotic protein in the Bcl-2 family implicated in lymphoid malignancies and solid tumors.^{42,43,56,57} Further studies to investigate other mechanisms by which Akt influences cell survival, such as regulation of caspases and transcription factors, as well as investigation of other signaling pathways, are warranted.^{58–63}

Finally, it should be noted that a role for Bcl-2 in the pathogenesis of Bcl-2-negative FL cannot be entirely excluded, as we were able to detect statistically significant levels of Bcl-2 protein by reverse phase protein microarray, despite negative staining by immunohistochemistry. This may be the result of the enhanced sensitivity of the protein array method. However, we cannot exclude the possibility that the low levels of Bcl-2 protein are due to admixed T cells, since slightly higher T-cell numbers were detected in Bcl-2-negative FL as compared with Bcl-2-positive FL (Table 1). In addition, we were able to identify the *BCL-2/JH* translocation by PCR in a small number of the Bcl-2 negative FL cases (Table 1). This percentage might have been higher if more sensitive methods, such as

fluorescence *in situ* hybridization, had been used.⁶⁴ We cannot rule out the possibility that Bcl-2 might have been more strongly expressed at other time points during the evolution of the disease, as variations in Bcl-2 expression over time may be seen. The mechanism of downregulation of Bcl-2 in the presence of t(14;18) is unknown, but a lack of correlation between the translocation status and protein expression has been reported previously.⁴¹

In conclusion, our study demonstrates striking similarities in the expression of apoptosis-related proteins in Bcl-2-negative and Bcl-2-positive FL. The increases noted in Bcl-XL suggest that this member of the Bcl-2 family may be compensating for the absence of Bcl-2 protein in some cases. These data support the hypothesis that most nodal FLs represent a single disease with a common final biochemical pathway. Our study did not include any cases of Grade IIIb FL, composed of centroblasts without centrocytes. Grade IIIb FL is rare, usually associated with diffuse areas, and is nearly always Bcl-2 protein negative. Recent studies have suggested differences in the molecular pathogenesis of Grade IIIb FL, linking it more closely to diffuse large B-cell lymphoma.^{65,66}

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