Quantitative Immunofluorescence Reveals the Signature of Active B-cell Receptor Signaling in Diffuse Large B-cell Lymphoma

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Abstract

**Purpose:** B-cell receptor (BCR)–mediated signaling is important in the pathogenesis of a subset of diffuse large B-cell lymphomas (DLBCL) and the BCR-associated kinases SYK and BTK have recently emerged as potential therapeutic targets. We sought to identify a signature of activated BCR signaling in DLBCL to aid the identification of tumors that may be most likely to respond to BCR-pathway inhibition.

**Experimental Design:** We applied quantitative immunofluorescence (qIF) using antibodies to phosphorylated forms of proximal BCR signaling kinases LYN, SYK, and BTK and antibody to BCR-associated transcription factor FOXO1 on BCR-cross-linked formalin-fixed paraffin-embedded (FFPE) DLBCL cell lines as a model system and on two clinical cohorts of FFPE DLBCL specimens (n = 154).

**Results:** A robust signature of active BCR signaling was identified and validated in BCR-cross-linked DLBCL cell lines and in 71/154 (46%) of the primary DLBCL patient specimens. Further analysis of the primary biopsy samples revealed increased nuclear exclusion of FOXO1 among DLBCL with qIF evidence of active BCR signaling compared with those without (P = 0.004). Nuclear exclusion of FOXO1 was also detected in a subset of DLBCL without evidence of proximal BCR signaling suggesting that alternative mechanisms for PI3K/AKT activation may mediate FOXO1 subcellular localization in these cases.

**Conclusion:** This study establishes the feasibility of detecting BCR activation in primary FFPE biopsy specimens of DLBCL. It lays a foundation for future dissection of signal transduction networks in DLBCL and provides a potential platform for evaluating individual tumors in patients receiving novel therapies targeting the BCR pathway. *Clin Cancer Res; 18(22); 6122–35. ©2012 AACR.*

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma, accounting for roughly 40% of all adult lymphoid malignancies and more than 80% of aggressive lymphomas (1, 2). DLBCL is heterogeneous in its biology and shows variable response to combination chemotherapy and anti-CD20 regimes. Prognosis is poor in approximately 50% of cases, indicating the need for more individualized therapeutic approaches targeting specific signaling pathways to further improve patient outcomes (3, 4).

B-cell receptor (BCR) expression and signaling are necessary for mature B-cell survival and there is increasing evidence for a critical role in lymphomagenesis (5–9). In B cells, the BCR signaling network is complex and involves the cross-activation and regulation of many signaling molecules. Stimulation of cell surface immunoglobulin (sIg) can occur by an antigen or occur independently of an exogenous ligand to transmit low-level tonic survival signals (9, 10). Stimulation leads to protein tyrosine kinase-mediated phosphorylation of the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) on the signaling subunit, a disulfide-linked Igo/lgb (CD79α/CD79β) heterodimer (10). Initial ITAM phosphorylation following receptor ligation is predominantly mediated by the Src-family kinase LYN that promotes subsequent recruitment and activation of the effector kinases SYK and BTK (11). SYK is a key player that couples BCR-mediated signals to downstream signaling pathways, and coordinate activation of LYN, SYK and BTK is required for proper BCR signal transduction (12–14). The limiting step in BTK activation appears to be generation of phosphatidylinositol 3,4,5-.....
Translational Relevance

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma with a poor prognosis in approximately 50% of patients. New therapeutic approaches are focused on targeting specific signaling pathways using small molecule inhibitors. B-cell receptor (BCR)–mediated signaling is an important pathway in DLBCL pathogenesis. In this work we evaluated BCR signaling using quantitative immunofluorescence (qIF) in paraffin-embedded DLBCL specimens and identified a robust signature based on the quantitation of phosphorylated proximal BCR signaling kinases LYN, SYK, and BTK. We detected active BCR signaling in almost 50% of patient tumors and found that this signaling translated into regulation of the proapoptotic transcription factor FOXO1. This study provides a foundation for dissection of signal transduction pathways in DLBCL and other malignancies using an immunostaining approach and implicates the use of qIF as a tool to identify patients with active BCR signaling that are receiving anti-BCR and other antitumor therapies.

response to BCR cross-linking with slg and established criteria to identify BCR pathway activation with a high degree of confidence. We extended this approach to patient specimens by conducting qIF on 2 primary DLBCL tissue microarrays (TMA) and observed coordinate phosphorylation of LYN, SYK, and BTK in 71/154 (46%) of human DLBCLs in situ.

We further sought to analyze the effect of proximal BCR signaling on the downstream target FOXO1 by qIF. We found that the majority of cell lines manifest high, constitutive activation of AKT and cytoplasmic FOXO1 that show a correlative increase upon slg cross-linking. In contrast, a range of FOXO1 subcellular localizations, from almost entirely nuclear to almost entirely cytoplasmic, was observed among primary DLBCLs in situ. Importantly, the degree of cytoplasmic FOXO1 was greater among primary DLBCL with coordinate activation of LYN, SYK, and BTK than those without ($P = 0.004$). These results reveal that a significant proportion of primary DLBCLs manifest robust activation of the proximal BCR signaling network that correlates with activation of the biologically significant downstream target FOXO1. We suggest that qIF provides a framework of assessing the integrity and activity of the BCR pathway in DLBCL biopsy samples that will assist in evaluating patients receiving targeted therapy.

Materials and Methods

Cell culture

The DLBCL cell lines SUDHL4 (DHL4), SUDHL6 (DHL6), OCI-LY4 (LY4), OCI-LY7 (LY7), Karpas-422 (K422), Pfeiffer, Toledo, HBL-1, and U2932 were cultured in RPMI 1640 medium (Mediatech) supplemented with 10% fetal calf serum and 2 mmol/L glutamine. The DLBCL cell line OCI-LY3 (LY3) was cultured in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 20% human serum (Gemini Bio-Products) and 2 mmol/L glutamine. All cells were maintained at 37°C in 5% CO₂. Cells were kindly provided at the time of study by the laboratory of Prof. Margaret Shipp (Dana-Farber Cancer Institute), and were originally obtained from the following sources: Pfeiffer and Toledo, American Tissue Culture Collection; SUDHL4, SUDHL6, and K422, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), and OCI-LY3, OCI-LY4 and OCI-LY7, Ontario Cancer Institute (The University of Toronto, Toronto, Ontario, Canada). Access to HBL-1 and U2932 were provided courtesy of Prof. Georg Lenz (Charité–Universitätsmedizin, Berlin).

The identities of the DLBCL cell lines used in this study were confirmed via short tandem repeat profiling (PowerPlex 1.2 system, Promega) and the online verification services of the cell banks Japanese Collection of Research Bioresources Cell Bank and DSMZ. Of note, a primary source genetic reference is not available for the cell lines OCI-LY3, OCI-LY4, and OCI-LY7 (the original cell line stock in Ontario has not been maintained), however the intralaboratory identity of these lines has been confirmed over time and the results of experiments using these cell lines are consistent with those previously reported (22).
BCR cross-linking
Cells (5 x 10⁶ in 0.5 mL RPMI) were stimulated with 10 μg/mL rabbit anti-human IgM (Jackson ImmunoResearch Laboratories Inc., cat. # 309-006-043) or goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., cat. # 109-006-006) for 10 minutes (22, 28). The cells were either lysed and used for qualitative Western blotting or fixed in formalin and used for cell pellet microarray construction.

Cell lysis and immunoblotting
The cells were lysed in NP-40 containing buffer supplemented with protease and phosphatase inhibitors as described previously (22). Protein concentrations were assessed by Bradford assay (Bio-Rad Laboratories, Inc.) and the samples were boiled for 3 minutes in 2× Laemmli sample buffer before loading onto a gel. Equal amounts of protein were separated by 4% to 15% SDS-PAGE (Bio-Rad Laboratories, Inc.) and transferred to Trans-Blot supported nitrocellulose membrane (Bio-Rad Laboratories, Inc.). Transferred proteins were visualized using Pierce’s staining (Sigma) and examined for equal loading. Nitrocellulose was rinsed with TBS/0.1% Tween 20 (TBS-T) and blocked with 5% milk powder or 5% FBS in TBS-T for 1 hour at room temperature. Nitrocellulose membranes were then incubated with following antibodies: antiphospho (pY396) LYN antibody in a 1:500 dilution (Epitomics, Inc., cat#1645-1), 1:1000 anti-LYN (Cell Signaling Technology, cat# 2796), 1:500 anti-phospho (pY223) SYK (Epitomics, Inc., cat# 2173-1), 1:1000 anti-SYK (Abcam, cat# ab3113), 1:500 anti-phospho (pY223) BTK (Novus Biologicals, Inc., cat# NR100-92487), and 1:1000 anti-BTK antibody (Abcam, cat# ab54219), 1:500 (RXXXS/T) antiphospho AKT substrate (Cell Signaling Technology, cat#9141), 1:1000 AKT1 antibody (Cell Signaling Technology, cat# 2938), antiphospho AKT (Cell Signaling Technology, cat# 9614), 1:1000 anti-FOXO1 antibody (Cell Signaling Technology, cat# 2880). To assess for equal loading, the blots were incubated with 1:500 anti-β-actin antibody (Santa Cruz Biotechnology, Inc., cat# sc-69879). The blots were stripped using Restore Plus Western Blot Stripping Buffer (Thermo Scientific, cat# 46430) and reprobed with a different antibody where indicated. After incubation with a primary antibody, the blots were washed 4 times for 15 minutes in TBS-T, followed by peroxidase-conjugated goat anti-rabbit IgG (Thermo Scientific, cat# 32460) or peroxidase-conjugated goat anti-mouse IgG (Thermo Scientific, cat# 32430) in a 1:500 dilution. All steps were then washed 4 times for 15 minutes in TBS-T, incubated with enhanced chemiluminescence (Amersham) chemoiluminescent substrate according to manufacturer’s instructions (Thermo Scientific, cat# 321), and exposed to autoradiography film (GE Healthcare).

Cell pellet microarrays
DLBCL cell lines, either untreated or cross-linked (22), were centrifuged in conical tubes for 5 to 10 minutes at 1,000 rpm. The media was removed and the cells were washed once with PBS. The cells were then pelleted by centrifugation and PBS was removed. The cell pellets were immediately resuspended in approximately 2 mL of 10% formalin and incubated at room temperature for 20 minutes. The formalin was removed and the cells were washed once with PBS, pelleted by centrifugation and resuspended in 1 mL PBS, transferred into 1.5 mL Eppendorf tube, and stored at 4°C. The cells were centrifuged for 10 minutes at 800 x g (2,900 rpm) and the supernatant was removed. Fifty to 60 μL of prewarmed Histogel (Richard-Allan Scientific) was added to each sample and the tubes were placed on ice to harden. The intact clots were then transferred to lens paper, placed in a histocassette, processed by standard methodologies overnight, and embedded in paraffin within a single block to form a cell pellet microarray. The experiments for each cell line were conducted at least in triplicate using independently treated cells.

TMA construction
Seventy-four patients with DLBCL diagnosed between 2004 and 2009 were selected from the files of the Brigham and Women’s Hospital (BWH, Table 1 and Supplementary Table S3) and 148 patients with DLBCL diagnosed between 2000 and 2006 from Massachusetts General Hospital (MGH), respectively, with Institutional Review Board approvals. Patients were classified according to the 2008 World Health Organization classification. TMA construction was conducted as described previously (29). In brief, tissue cylinders with a diameter of 0.6 mm were punched from representative regions from each donor tissue block and brought into a recipient paraffin block using a semiautomatic robotic precision instrument. Three 0.6 mm cores of DLBCL were arrayed from each case.

Immunohistochemistry
Chromogenic and immunofluorescent immunohistochemistry (IHC) was conducted on DLBCL cell pellet microarrays and TMAs using 5 μm-thick sections on individual fresh-cut slides. We tested numerous antiphospho-LYN, SYK, and BTK antibodies under a wide range of conditions against untreated or slg-cross-linked formalin-fixed, paraffin-embedded (FFPE) cell lines to identify the best reagent for IHC using FFPE tissue samples, comparing

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results by IHC to Western blot analyses of cell lysates under the same stimulation conditions and using the same antibodies. On the basis of this systematic approach we found the antibodies and procedures below gave optimal performance and reproducibility in qIF compared with others.

Slides were baked, soaked in xylene, passed through graded alcohols, and then pretreated with either DAKO pH 9 retrieval solution (DAKO USA) for pLYN, pSYK, and pBTK; 1 mmol/L EDTA, pH 8 (Zymed) for FOXO1; 10 mmol/L citrate, pH 6 for (RXXS/T') anti-phospho AKT substrate in a steam pressure cooker (Decloaking Chamber; BioCare Medical) as per manufacturer’s instructions followed by washing in distilled water. All further steps were done at room temperature in a hydrated chamber. Slides were then treated with peroxidase block (DAKO) for 5 minutes to quench endogenous peroxidase activity. Staining was conducted with 1:600 anti-pY396LYN, 1:100 anti-pY323SYK, 1:25 anti-pY551BTK (Epitomics, Inc., cat#1685-1), 1:100 anti-FOXO1, and 1:2500 (RXXS/T') anti-phospho AKT substrate antibodies for 1 hour. Where indicated, double staining with CD20 antibody (clone L26; DAKO, ready to use solution) was conducted. For immunofluorescent staining, slides were washed in 50 mmol/L Tris-HCl (pH 7.4) then labeled with diluted 1:200 Alexa 555 goat anti-rabbit (phosphomarkers, FOXO1) and Texas Red-conjugated goat anti-mouse (CD20; Molecular Probes) for 30 minutes and coverslipped using ProlongGold antifade (Life Technologies) for 30 minutes and coverslipped using ProlongGold antifade (Life Technologies). Immunoperoxidase staining was developed using a diaminobenzidine (DAB) chromogen kit (DAKO) as per the manufacturer and counterstained with Harris hematoxylin (Polyscientific).

Quantification of immunofluorescence staining

The fluorescently stained slides were scanned using digital TissueFAXS imaging system and evaluated using TissueQuest imaging analysis software (TissueGnostics). In brief, single cells were identified using an algorithm based on a preselected so-called master channel (based on nuclear DAPI staining) and the intensity of staining within either a cytoplasmic or total cell mask was quantified (Supplementary Figs. S1 and S3). For cell line analysis the mean staining intensity of the phospho-markers (pLYN, pSYK, pBTK, pAKT-substrate) and FOXO1 was plotted against mean DAPI intensity. For the TMA analysis, the slides were double stained with a pan B cell marker CD20 along with either pLYN, pSYK, pBTK, or FOXO1 and the mean intensity of a phospho-marker/FOXO1 staining was plotted against mean intensity of CD20 to determine the percentage of double positive cells. Positive cells were selected based on the cutoff values that were set by engaging negative controls and the backward and forward gating tool of the software. The results of the analysis are depicted in scattergrams (dot-plots) similar to those used in flow cytometry.

Statistical analysis

A combined score for %pSYK+ and %pBTK+ was derived from linear regression using a maximum-likelihood algorithm (30) using the formula (pSYK,pBTK) = (pSYK + mpBTK)/(1 + m), where m is the slope of the fitted line and (pSYK,pBTK) has a range of 0 to 100. Estimates of statistical significance were based on the 2-tailed student t test for continuous variables or the binomial distribution for discrete categories. For analysis of FOXO1 localization, the FOXO1 score \( F_{\text{score}} = 100 \times \text{FOXO1}_{\text{cyt}}/\text{FOXO1}_{\text{tot}} \) was used, being the percentage ratio of FOXO1+ cells using either a cytoplasmic (FOXO1cyt) or total cell (FOXO1tot) mask.

For the analysis of TMA data, a series of selection criteria were applied for inclusion of the specimen in the total sample (i) CD20+ > 10% and >5% double positives for any core, (ii) where 3 cores were analyzable, reject those >40% different from the other 2, (iii) ≥2 of 3 cores, subject to these exclusions, analyzable by Tissuequest for each of pLYN, pSYK-pBTK (paired), and FOXO1. Of 74 specimens within the BWH array, 60 fulfilled all criteria and are included in the final analysis. A validation TMA consisting of 148 cases was analyzed by the same selection criteria; 94 fulfilled all criteria and were included in final analysis. Further details and data tables are provided as Supplementary Information.

Results

Immunohistochemical signature for active BCR signaling in DLBCL cell lines

A panel of phospho-specific antibodies was developed to detect active BCR signaling by immunofluorescence of previously characterized DLBCL cell lines embedded in a cell pellet microarray. BCR activation was stimulated by cross-linking sIg with anti-immunoglobulin antibodies before fixation. We selected 3 phospho-specific antibodies directed toward known markers of BCR activation, LYN pY396 (pLYN; ref. 31, 32), SYK pY323 (pSYK; ref. 33, 34), and BTK pY551 (pBTK; ref. 35, 36). Results for cell lines LY7, DHL4, LY3, and Toledo are shown (Fig. 1A). Cross-linking of sIg led to a significant coordinate increase in phosphorylation of LYN, SYK, and BTK over baseline levels in LY7, DHL4, and LY3 but not in Toledo. Similar results were obtained by chromogenic staining (Supplementary Fig. S1).

Quantitative analysis of pLYN, pSYK, and pBTK levels was conducted using the TissueFAXS imaging system and Tissuequest software (Supplementary Fig. S2 and Supplementary Methods). The percentage positive cells %pLYN+, %pSYK+, and %pBTK+ (Fig. 1B) agreed qualitatively with independent visual assessment by 2 expert pathologists (data not shown); qualitative Western blotting supports the effect of cross-linking (Supplementary Fig. S3). In subsequent analysis we use the percentage positive cells as independent variables (i.e., pLYN = %pLYN+, pSYK = %pSYK+, pBTK = %pBTK+).

qIF analysis of DLBCL cell lines reveals a metric for BCR activation

We next analyzed the percentage of pLYN+, pSYK+, and pBTK+ cells from 3 independent experiments in a panel of 10 cell lines. To assess the diagnostic potential of pLYN, pSYK, and pBTK, we compared the difference between mean
values of the 3 phosphomarkers for untreated and cross-linked samples in each cell line (Supplementary Table S1). Although pLYN had the highest levels upon cross-linking, intermediate levels were also observed in 50% of untreated samples (DHL4, DHL6, HBL-1, LY3, and Pfeiffer). In contrast, pSYK and pBTK had very low or undetectable levels in all untreated samples that increased significantly upon cross-linking and therefore better predictors of sIg-mediated BCR activation than pLYN.

Of the 3 phosphomarkers pSYK is the strongest single predictor of BCR cross-linking. Five of 10 cell lines (LY7, U2932, DHL4, DHL6, HBL-1) displayed pSYK < 10 in uncross-linked specimens and pSYK > 50 in cross-linked specimens and a significant difference between the average values (P ≤ 0.06, Supplementary Table S1, Fig. 2A). These cell lines, referred to as Group I, comprise 3 BCR/germinal center B-cell (GCB) type (LY7, DHL4, DHL6) and 2 ABC type (U2932, HBL-1) cell lines according to previous molecular profiling studies, although of the other 5 cell lines, referred to as Group II, 4 are of the OxPhos type (Pfeiffer, Toledo, K422, LY4) cell lines (Supplementary Table S2). Group II included 1 BCR/ABC type cell line (LY3), for which the increase upon cross-linking was statistically significant for pLYN but did not reach statistical significance for pSYK.

Figure 1. qIF of proximal BCR signaling in DLBCL cell lines. A, immunofluorescent staining of paraffin-embedded DLBCL cell lines LY7, DHL4, LY3, and Toledo, untreated and BCR cross-linked, with anti-pLYN (Y396; green), anti-pSYK (Y323; red), and anti-pBTK (Y551; orange). Nuclei stained with DAPI (blue). Increased phosphorylation upon cross-linking is observed for LY7, DHL4, and LY3. No significant phosphorylation is observed for Toledo in either untreated or cross-linked cells. B, scatterplot of total intensity per cell for phosphomarkers (Texas Red) versus nuclear intensity (DAPI) from Tissuequest analysis of cells shown in A. The value displayed in the top right corner of each scatterplot is the % positive (average of 3 independent experiments). Quantitative analysis agrees well with qualitative visual assessment. Note: cells negative for staining lie along the y-axis of the scatterplot.
or pBTK by qIF (pSYK untreated 0.8 ± 0.7, cross-linked 7.8 ± 4.9, \( P = 0.13 \)). Overall Group I showed high levels for all 3 phosphomarkers upon cross-linking; although Group II had negligible or more variable levels of the 3 phosphomarkers upon cross-linking: only Group I cell lines showed a significant increase in at least 2 of the 3 phosphomarkers studied following BCR cross-linking. These results largely, but not completely, recapitulate changes in phosphomarkers observed by Western blotting as previously reported (22).

As pSYK and pBTK are well correlated (Fig. 2B), we derived a weighted average (\( \text{pSYK}_{w}, \text{pBTK} \)) (see Supplementary Methods) and compared the average value for Group I cross-linked and untreated samples (Fig. 2C). Both pSYK and (\( \text{pSYK}, \text{pBTK} \)) discriminate cross-linked from untreated samples with high statistical significance. Thus, we selected \( \text{pSYK}_{w}, \text{pBTK} \) as a criterion of BCR activation incorporating 2 independent tests of BCR signaling. A value of \( \text{pSYK}, \text{pBTK} \geq 15 \) should be a strong indicator of proximal BCR pathway activation (BCR\(^{+} \)) in DLBCLs for which Group I cell lines are appropriate models. All untreated samples as well as crosslinked Group II cell lines except Pfeiffer are \( \text{pSYK}, \text{pBTK} \) (BCR\(^{+} \)) indicating undetectable proximal BCR pathway activation by qIF using this criterion.

Plotting \( \text{pSYK}, \text{pBTK} \) versus plYN for all samples in Group I (Fig. 2D), we further noted that all samples with \( \text{pSYK}, \text{pBTK} > 15 \) also had plYN > 15, consistent with the hypothesis that phosphorylation of SYK and BTK (plYN = 74 ± 13 for cross-linked Group I). Hence, \( \text{pSYK}, \text{pBTK} > 15, \text{plYN}>15 \) are appropriate criteria for detecting proximal BCR pathway activation in DLBCLs without falsely identifying BCR activation in any DLBCLs for which the cell line panel is representative.

**qIF analysis of BCR signaling in a patient DLBCL TMA**

We proceeded to analyze pLYN, pSYK, and pBTK levels by qIF in a TMA composed of 3 cores each from 60 primary DLBCL patient specimens. Aggregate clinical statistics are provided in Table 1 and summaries of all cases in Supplementary Table S3. In contrast to DLBCL cell lines, specimens in the TMA contain additional cell types. Therefore, each phosphomarker was probed alongside the B cell-specific marker CD20, and the percentage double-positives recorded (\( \text{pLYN} = \% \text{pLYN} \cdot \text{CD20} \), \( \text{pSYK} = \% \text{pSYK} \cdot \text{CD20} \), \( \text{pBTK} = \% \text{pBTK} \cdot \text{CD20} \)).

The percentage CD20\(^{+} \) cells across the TMA was high (73 ± 27%) and was highly correlated for each core between different slides (\( \Delta \% \text{CD20}^{+} \) = 5 ± 4%). Visual inspection confirmed that the vast majority (>90%) of CD20\(^{+} \) cells are large in size with variably pleomorphic nuclei consistent with lymphoma. Results for all specimens are reported in Supplementary Table S4. The standard deviation between cores of the same specimen gives some measure of spatial heterogeneity within patient samples. The average error was 7.9%, 7.4%, and 7.7% for pLYN, pSYK, and pBTK, respectively. Excluding samples with average less than the average error, the relative error (\( \Delta Z/Z \)) was 31%, 45%, and 40% for plYN, pSYK, and pBTK, respectively. Levels of pBTK versus pSYK were higher in the primary DLBCL TMA than the cell pellet microarray (\( \text{m}_{\text{BMA}} = 0.895 \), Fig. 3 inset). Hence, the criterion (pSYK, pBTK) = 15 derived from cell pellet microarray was renormalized to (pSYK, pBTK) = 14.2 in the primary DLBCL TMA (see Supplementary Methods). The value of (pSYK, pBTK)
Figure 3. Quantitative assessment of active BCR signaling in primary DLBCL tumors. Scatterplot of pSYK, pBTK versus pLYN for primary DLBCL patient specimens in a TMA analyzed by qIF for pLYN (Y396), pSYK (Y323), and pBTK (Y551) and CD20. Dashed lines represent criteria for active BCR signaling (pSYK, pBTK > 14.2, pLYN > 15). Specimens are categorized as pSYK, pBTK+/pLYN+ = BCR+ (yellow), pSYK, pBTK+/pLYN- (orange), pSYK, pBTK-/CD20+ (magenta), or pSYK, pBTK-/CD20-/pLYN- (blue). Representative fields of view for 3 BCR phosphomarkers (green) and CD20 (red) are shown for selected specimens, clockwise from top right (i) strong BCR+, (ii) intermediate BCR+, (iii) pSYK, pBTK+/pLYN-, (iv) BCR-, (v) pSYK, pBTK+/pLYN- (nuclear pSYK). Inset, scatterplot of pBTK versus pSYK for qIF analysis of DLBCL patient specimens, Pearson correlation coefficient r = 0.81.
versus pLYN were plotted (Fig. 3) and cases evaluated by the criteria \( p_{\text{SYK}, p_{\text{BTK}}} = 14.2, \) \( p_{\text{LYN}} = 15. \)

Almost half the cases (27/60, 45%) are \( p_{\text{SYK}, p_{\text{BTK}}} \) \( p_{\text{LYN}} \), or BCR . Hence, almost half the primary DLBCL cases examined had levels of phosphorylated BCR-dependent kinases consistent with proximal BCR pathway activation. Representative fields-of-view for the 3 phospho-markers for 2 typical BCR cases (Fig. 3, right) reveal a punctate membrane pattern for pLYN, pSYK, and pBTK suggestive of BCR clustering at the cell surface. Similar findings were recently observed in ABC-type primary DLBCLs (21). Conversely, almost one-quarter of cases (14/60, 23%) matched the criteria \( p_{\text{SYK}, p_{\text{BTK}}} \) \( p_{\text{LYN}} \), or BCR . Visual assessment of these cases (Fig. 3, lower left) confirmed these cases as true staining negatives. Results of chromogenic assessment of these cases (Fig. 3, lower left) confirmed these cases as true staining negatives. Results of chromogenic staining were in qualitative agreement with immunofluorescent analysis (Supplementary Fig. S5), however, CD20 cannot be simultaneously analyzed by this method of detection.

One-quarter of cases (15/60, 25%) matched the criteria \( p_{\text{SYK}, p_{\text{BTK}}} \) \( p_{\text{LYN}} \). Such cases are not observed in DLBCL cell lines nor are they consistent with the biologic function of LYN upstream of SYK/BTK. Visual assessment revealed that pSYK was predominantly nuclear in some of these cases (Fig. 3, top left). Nuclear localization of SYK has been previously described in B cells and squamous cell carcinoma and has been linked to alternate splicing and transcriptional repressor function (37–40). In the present context, however, such cases are inconsistent with the majority of \( p_{\text{SYK}, p_{\text{BTK}}} \) \( p_{\text{LYN}} \) cases and were not designated positive for BCR signaling. The influence of nuclear pSYK staining was minimized by use of a cytoplasmic mask.

### qIF analysis of BCR-mediated AKT activity and FOXO1 subcellular localization in DLBCL cell lines

Proximal BCR pathway activation in DLBCL TMA specimens cannot be controlled by manipulation of sIg as for the cross-linked samples in the DLBCL cell pellet microarray. Also, unlike the cell pellet microarray, patient samples are subject to numerous preanalytical variables, notably time to fixation that may significantly impact the detection of phosphoepitopes (41). Thus, we extended our analysis on primary tissues to BCR-mediated downstream signaling molecules (15, 42) to further validate our assignment of cases as BCR and BCR and to show the detection of a complete signal transduction network in individual patient specimens. AKT phosphorylation is difficult to accurately assess in clinical specimens because of rapid antigen degradation by dephosphorylation (41, 43, 44). BCR-mediated activation of AKT, however, promotes the phosphorylation of FOXO1 and its translocation from the nucleus to the cytoplasm; hence we anticipated that BCR DLBCLs would have a significantly higher proportion of cytoplasmic FOXO1 than BCR cases. Therefore, we analyzed the DLBCL cell line panel for FOXO1. To quantitatively assess the degree of FOXO1 cytoplasmic localization, we defined a FOXO1 score \( F_{\text{SY}} = 100 \times \frac{\text{FOXO1}_{\text{cyt}}}{\text{FOXO1}_{\text{tot}}} \), being the percentage ratio of FOXO1 cells using either a total cell (FOXO1 tot ) or cytoplasmic (FOXO1 cyt ) mask (Supplementary Fig. S5 and Supplementary Methods).

Results for IHC of 3 representative cell lines (U2932, DHL6, and Toledo) are shown and correlated with qIF analysis of percentage positive cells for FOXO1 using a cytoplasmic mask (Fig. 4A). U2932 had low basal levels of FOXO1 cyt and a significant increase upon cross-linking. DHL6 had high basal levels and only a minor increase upon stimulation, whereas Toledo had constitutively high FOXO1 cyt and no significant increase upon stimulation. Chromogenic staining was in qualitative agreement with immunofluorescent analysis (Supplementary Fig. S6A) but quantitative delineation of the cytoplasm and extent of cytoplasmic localization was not possible by this detection method. In analysis of 3 independent experiments for all DLBCL cell lines, U2932 and HBL1 showed highly significant cytoplasmic localization of FOXO1 upon cross-linking, but the remaining DLBCL cell lines had constitutive cytoplasmic localization and increases upon cross-linking were not statistically significant (Supplementary Table S3, Supplementary Fig. S7C).

Constitutive FOXO1 cytoplasmic localization may be either selected for or induced by the conditions of \textit{in vitro} cell culture, yet FOXO1 localization may still be well correlated with AKT activation. We therefore assessed the level of AKT activation in the DLBCL panel using a phosphospecific AKT substrate antibody \( p_{\text{AKT sub}} \) that detects both autophosphorylated AKT and phosphorylated AKT substrates. Qualitative Western blotting (Fig. 4B) supports the detection of autophosphorylated AKT by \( p_{\text{AKT sub}} \) and qIF analysis (Supplementary Fig. S7A) confirmed increased AKT activity upon cross-linking. Analysis of 3 independent experiments for all DLBCL cell lines revealed a statistically significant increase in \%pAKT sub cells for most cell lines (Supplementary Table S5, Supplementary Fig. S7C). However, \%pAKT sub levels did not correlate with BCR phospho-markers across the panel, reflecting a degree of independence between the BCR and PI3K/AKT pathways in the cell lines. Nevertheless, changes in \%pAKT sub and FOXO1 cytoplasmic localization were correlated as evidenced by plotting the difference between cross-linked and untreated samples for the FOXO1 score \( \Delta F_{\text{SY}} \) versus \( p_{\text{AKT sub}} \) (Fig. 4C, Supplementary Fig. S7C). Hence, FOXO1 cytoplasmic localization is a valid surrogate marker of AKT activation in DLBCL.

### Correlation of BCR signaling and FOXO1 localization in a patient DLBCL TMA

Based on the correlation between AKT activity and FOXO1 localization, we proceeded to analyze the patient DLBCL TMA with FOXO1 and CD20 antibodies.
percentage double-positives, %FOXO1 CD20+, was determined using both a total cell and cytoplasmic mask and the FOXO1 score (F_cyt) calculated as for the DLBCL cell lines. Use of the F_cyt score was validated in comparison of calculated F_cyt values for each case with visual scoring conducted independently by 2 expert pathologists using (i) fluorescent staining and a 3-tier scoring system, and (ii) chromogenic (DAB) staining and a 4-tier scoring system (Supplementary Fig. S8). Mean F_cyt scores correlated quantitatively with both expert assessments.

The FOXO1 scores F_cyt for all 60 primary DLBCL patient specimens were compared with their previous evaluation of BCR signaling (Supplementary Table S4, Supplementary Fig. S9). A representative BCR “F_cyt+” and BCR “F_cyt-” case are shown and correlated with qIF analysis of positivity for FOXO1 using both cytoplasmic and whole cell masks (Fig. 5A). To test our criteria for active BCR signaling, we compared the distribution of F_cyt values for (pSYK, pBTK) “pLYN” (BCR+) and (pSYK, pBTK) “pLYN” (BCR-) cases (Fig. 5B). The mean F_cyt score for BCR+ cases was significantly higher than for BCR- cases (57 ± 32 vs. 28 ± 25, P = 0.004). No statistically significant difference in F_cyt score was observed for (pSYK, pBTK) “pLYN”- and (pSYK, pBTK) “pLYN”- subsets compared with BCR+ cases.

Analysis of clinical correlates

We compared our qIF analysis for pLYN, (pSYK, pBTK), and F_cyt to clinical data available for 60 patient specimens. There was no correlation between qIF variables and patient age, tumor location, or proliferation index. A significant correlation was noted on the basis of sex however; females had higher values of (pSYK,pBTK) than males (35 ± 29 vs. 18 ± 23; P = 0.01). Two-thirds of BCR+ cases (18/27, 67%) were female compared with almost equal ratios for all specimens (32/60, 53%).

According to the cell of origin profiling system, activated B-cell (ABC)–type tumors have significantly decreased survival versus GCB-type tumors (45), and a significant number of ABC-type DLBCLs are reported to rely on “chronic active” BCR signaling to mediate survival (21). Similarly, the consensus cluster profiling system identifies a subset of BCR-type tumors that are reported to rely on tonic BCR signaling for survival (22, 23). We therefore analyzed staining by CD10, BCL6, and MUM1 and applied the algorithm of Hans and colleagues to all 60 cases (46). Overall, 35 cases were assigned “GCB” and 25 “non-GCB.” We found no difference between any qIF variable for GCB versus non-GCB specimens over all cases, nor any significant enrichment in GCB or non-GCB cases in the subset of BCR+ or BCR- cases from the overall distribution (Supplementary Tables S3 and S4).

Independent cohort validation

To confirm that our qIF analysis is not biased by patient selection or sample preparation procedures specific to one institution (BWH), we repeated our analysis on a validation
matched the criteria (pSYK, pBTK)\(^+\)pLYN\(^+\) and only a small number of cases (5/94, 5%) matched the criteria (pSYK, pBTK)\(^+\)pLYN\(^-\).

The difference between the mean \(F\text{cyt}\) score for BCR\(^+\) and BCR\(^-\) cases in the MGH TMA was highly significant (70 ± 25 vs. 34 ± 23, \(P < 10^{-6}\), Fig. 6C). We further analyzed staining by CD10, BCL6, and MUM1 and applied the algorithm of Hans and colleagues to all 94 cases (46). Equal numbers (47 cases each) were assigned “GCB” and “non-GCB.” We found no difference between any phosphomarker for GCB versus non-GCB specimens over all 94 patient specimens nor any significant variation in GCB or non-GCB cases in the subset of BCR\(^-\) or BCR\(^+\) cases from the overall distribution, although GCB cases had a higher average value of \(F\text{cyt}\) than non-GCB cases (61 ± 29 vs. 44 ± 28, \(P = 0.008\)). In summary, our analysis of an independent cohort validates the results of our qIF analysis, with a similar distribution of cases, a clear correlation between BCR\(^-\) cases and increased \(F\text{cyt}\) values, and lack of correlation between qIF analysis of BCR phosphomarkers and IHC-based molecular profiling.

**Discussion**

This study establishes the use of qIF to identify proximal BCR activation in FFPE biopsy samples of DLBCL. From quantitative analysis of the phosphorylated kinases LYN, SYK, and BTK and the weighted average (pSYK, pBTK), we developed and validated criteria for identifying proximal BCR signaling in DLBCL cell lines. When applied to 2 independent patient TMAs, this analysis revealed that almost half of patient specimens exhibit a signature of proximal BCR signaling (BCR\(^-\)) in situ. We also found that proximal BCR signaling correlates with increased cytoplasmic localization of the prosurvival transcription factor FOXO1, further suggesting a key role for BCR signaling in the activation of the PI3K/AKT/FOXO1 survival pathway in vivo. Surprisingly, we found no correlation between our qIF analysis of BCR signaling and IHC-based molecular profiling by the Hans’ algorithm. We note however, that a similar lack of correlation was observed in a separate, recent study (24).

A major concern with phospho-specific epitopes in primary patient specimens is the influence of preanalytical variables, especially the time to fixation that is uncontrolled in a retrospective analysis of paraffin-embedded specimens. Preliminary results on the spleens of mice following the introduction of DLBCL xenografts (LY1 cell line, data not shown) suggest that BCR phosphomarkers used in this study remain detectable by chromogenic staining following at least 30 minutes delay before fixation. Previous studies determined the half-life of FOXO1 to be at least 1 hour in HepG2 and HEK293T cells (47, 48). The similarity of our results on TMAs from 2 separate institutions suggests that errors arising from preanalytical variables will not significantly distort qIF analysis of BCR signaling in multicenter studies. Separate institutions may use common procedures giving increase to the same systematic error however, so prospective studies of preanalytical variables in the context of primary human specimens using similar detection protocols as described here are required to validate the qIF
method. Notwithstanding these limitations however, our results reveal that at minimum, approximately one-half of primary patient specimens contain detectable levels of pLYN, pSYK, pBTK, and FOXO1 even in the absence of stringent preanalytical protocols.

Although activation of the BCR signaling cascade is a common feature of DLBCL in situ, we emphasize that the biologic significance of these results still needs to be confirmed. Of note, we observed increased levels of pSYK and pBTK following cross-linking sIg in the GCB cell lines DHL4 and DHL6. Although in agreement with previous results from Western blotting of these cell lines following stimulation (22), and evidence that DHL4 and DHL6 are sensitive to chemical inhibitors of SYK (22, 24), DHL4 and DHL6 are unaffected by shRNA-mediated knockdown of CD79 or SYK (21), implying that these cell lines do not rely on "chronic active" BCR signaling for survival. Thus, it is likely that the detection of an inducible BCR signaling cascade is necessary but not sufficient for determining if a cell line requires BCR signaling for survival in the absence of functional data. Similarly, determining whether qIF analysis can serve to predict if an individual patient’s tumor is dependent on BCR signaling for growth and survival will require studies correlating the BCR score with clinical response in trials using directed therapies.

An unanticipated finding of our study was the detection of pLYN without detection of pSYK or pBTK in unstimulated GCB cell lines DHL4 and DHL6 and in 12% to 25% of primary DLBCL specimens. This precludes the use of pLYN as a primary criterion for BCR-specific signaling, although it is a useful secondary criterion, i.e., pLYN is necessary but not sufficient for a tumor to be classified as BCR⁺. The unique dual role of LYN in B cells beyond the activation of SYK and BTK is well documented, including negative regulatory roles in BCR signaling via phosphorylation of immunoreceptor tyrosine based inhibition motifs in CD22, CD72, PIR-B, and FcyRIB (51, 52). Consistent with the notion that LYN has additional roles in tumor cell survival, the GCB cell lines OCI-Ly7 and OCI-Ly19, which were resistant to BTK shRNAs, were sensitive to 1 of 3 LYN shRNAs (NM_002350_bp1462) tested by Davis and colleagues (21). The biologic significance of LYN phosphorylation in DLBCL is a topic of future investigation.

Figure 6. Validation of qIF in an independent cohort of primary DLBCL tumors. A, scatterplot of pBTK versus pSYK for qIF analysis of MGH DLBCL TMA, Pearson correlation coefficient r = 0.76. B, scatterplot of pSYK,pBTK versus pLYN for MGH DLBCL TMA analyzed by qIF for pLYN (Y396), pSYK (Y323), and pBTK (Y551) and CD20. Dashed lines represent criteria for active BCR signaling (pSYK,pBTK > 14.2, pLYN > 15). Specimens are categorized as (pSYK,pBTK, pLYN) = BCR⁺ (yellow), (pSYK,pBTK, pLYN) = orange, (pSYK,pBTK, pLYN) = magenta, or (pSYK,pBTK, pLYN) = BCR⁻ (blue). C, plot of F cyt scores (*) for BCR⁺ and BCR⁻ cases in the MGH DLBCL TMA. —, arithmetic mean.
We propose that the application of qIF represents a valuable new tool to complement molecular profiling studies and flow cytometry in the analysis of tumor-associated signaling pathways. In contrast to transcriptional profiling, qIF enables the analysis of posttranslational modifications and subcellular localization of the expressed targets within a heterogeneous population of cells, which are not readily addressed by mRNA-based gene expression studies. Although flow cytometry can be used to detect posttranslational modifications and analyze heterogeneous cell populations, intracellular markers are technically demanding and abnormally large tumor cells may go undetected due to either cell loss during processing or a paucity of neoplastic cells. Allocation of appropriate material for flow cytometric studies can also pose a challenge as neoplastic infiltrates may not involve the entire specimen and evaluation of the signaling molecules in situ using qIF offers an additional advantage. Finally, we show that qIF can successfully analyze the functional state of a signal transduction cascade in paraffin-embedded tissue, which is frequently the only patient material available for evaluation by the pathologist.

The use of qIF to analyze the functional state and localization of signaling components in routine clinical specimens is of great value for comparison with results obtained from model systems or cell lines. In this study alone 3 quantitative differences were observed between DLBCL cell lines and tissue specimens: (i) the level of pBTK relative to pSYK in tissue specimens was twice that observed in DLBCL cell lines, (ii) FOXO1 localization varied almost the entire range Fcy,0–100 in tissue specimens, but was significantly cytoplasmic (Fcy,>50) in the majority of DLBCL cell lines, (iii) pLYN levels were highly correlated with FOXO1 cytoplasmic localization in tissue specimens suggesting it was a significant indicator of BCR signaling, whereas several unstimulated DLBCL cell lines had significant levels of pLYN suggesting it was a poor primary indicator of BCR signaling. This illustrates both the importance of validating molecular hypotheses based on model systems in primary tissue samples and the value of qIF analysis in conducting such validation studies.

In addition to the BCR/PI3K/AKT pathways examined in this study, other survival pathways implicated in DLBCL include constitutive activation of NF-κB via B-cell activation factor (BAFF)-R (BR3; 53), p38-activated protein kinase (mitogen-activated protein kinase) expression (54) or Hedgehog signaling pathways (55). Future qIF studies combining phospho-specific antibodies to critical signaling molecules in these additional signaling pathways could address their contribution to DLBCL biology in situ. Moreover, the development of qIF-based signatures for these and other components could provide a means of comprehensive analysis of the molecular basis of BCR-mediated signaling and survival in DLBCL tumors.

In conclusion, we have developed a tissue-based qIF approach for analyzing components of the proximal BCR and distal AKT/FOXO1 signaling pathways in FFPE tissue. Quantification of signaling proteins in tissue shall be of great value in identifying future therapeutic targets in DLBCL and other aggressive lymphomas and in correlating the therapeutic efficacy of inhibitors of the BCR and PI3K/AKT pathways. Moreover, qIF-based classification of DLBCL may aid in understanding of the basic differences in the biology of these tumors and their variable response to treatment. The technology and analytical approach presented here can be used as a basis for dissection of activity of signaling pathways in many different hematolymphoid and non-hematolymphoid malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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