B Cells With High Side Scatter Parameter by Flow Cytometry Correlate With Inferior Survival in Diffuse Large B-Cell Lymphoma

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Abstract

Despite advances in the understanding of diffuse large B-cell lymphoma (DLBCL) biology, only the clinically based International Prognostic Index (IPI) is used routinely for risk stratification at diagnosis. To find novel prognostic markers, we analyzed flow cytometric data from 229 diagnostic DLBCL samples using an automated multiparameter data analysis approach developed in our laboratory. By using the developed automated data analysis pipeline, we identified 71 of 229 cases as having more than 35% B cells with a high side scatter (SSC) profile, a parameter reflecting internal cellular complexity. This high SSC B-cell feature was associated with inferior overall and progression-free survival (P = .001 and P = .01, respectively) and remained a significant predictor of overall survival in multivariate Cox regression analysis (IPI, P = .001; high SSC, P = .004; rituximab, P = .53).

This study suggests that high SSC among B cells may serve as a useful biomarker to identify patients with DLBCL at high risk for relapse. This is of particular interest because this biomarker is readily available in most clinical laboratories without significant alteration to existing routine diagnostic strategies or incurring additional costs.

Diffuse large B-cell lymphoma (DLBCL) is an aggressive malignancy of mature B lymphocytes representing about 40% of non-Hodgkin lymphomas.1 Although DLBCL represents a single histopathologic diagnosis, it is heterogeneous in terms of morphologic features, immunophenotypic profiles, cytogenetic findings, and clinical outcomes.2-7 Prognostic markers include the International Prognostic Index (IPI), BCL2 and BCL6 protein expression, and DLBCL molecular subtypes (activated B-cell vs germinal center B cell [GCB]).7,10 However, BCL2 and BCL6 are no longer relevant in the rituximab era, and defining molecular subtypes by immunohistochemical analysis may not be sufficiently sensitive or reproducible across different laboratories.11-14 Therefore, new biomarkers that can risk-stratify DLBCL with the current standard of therapy and be easily implemented in clinical laboratories are needed.

Flow cytometric (FCM) immunophenotyping is a standard component in the diagnosis and monitoring of lymphoproliferative disorders. It is quantitative and can simultaneously assess multiple parameters in thousands of individual cells. We have recently demonstrated that FCM studies can provide useful prognostic information beyond qualitative assessment for clonality and aberrant antigen expression.15 A number of other studies have also been conducted to identify surface immunophenotypic features that correlate with survival of patients with DLBCL (for review, see Wu and Keating16).

It is widely recognized that data analysis is by far one of the most challenging and time-consuming aspects of FCM experiments.17-28 Gating FCM data is a highly subjective, time-consuming, and often imprecise process in which the investigators manually determine the regions in 1- or 2-dimensional space that contain the interesting data, based
on their knowledge of the experimental factors and experience. Identifying the important information within FCM data is challenging using ordinary manual gating of the data. In the situations in which there is no knowledge about where to search for a potentially interesting marker, one needs to exhaustively search all possible 1- and 2-dimensional representations of the FCM data and examine the prognostic significance of the identified markers. This task is challenging, especially when these data are high dimensional.

In an attempt to discover additional features in the data that may be of clinical relevance, we developed and applied an automated pattern-discovery algorithm to identify discrete cell populations that cluster lymphoma samples according to their overall similarities based on multiple immunophenotypic and cellular characteristics by FCM studies. We identified that orthogonal, or “side” scatter (SSC) by CD19+ B cells is highly correlated with clinical outcome, independent of IPI significance of the identified markers. This task is challenging, even when these data are high dimensional.

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Materials and Methods

Case Selection

We included the data for 229 patients with de novo DLBCL, referred to as cohort A, diagnosed according to the 2008 World Health Organization criteria1 who had FCM analysis performed on diagnostic biopsy specimens between 1997 and 2007. Patients were older than 18 years, HIV−, and treated with curative intent using cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) with or without rituximab (R), an antibody directed against the CD20 antigen. A follow-up study of an additional 33 cases of de novo DLBCL, referred to as cohort A, diagnosed between March and November 2009 was done using 8-color FCM studies. All patient samples were recruited without any other formal inclusion or exclusion criteria. Ethical approval to conduct this retrospective review was granted by the University of British Columbia–British Columbia Cancer Agency Institutional Review Ethics Board, and informed consent was obtained in accordance with the Declaration of Helsinki.

Monoclonal Antibodies

Cohort A

Cell suspensions from freshly disaggregated lymph node biopsies were stained with monoclonal antibodies conjugated to FITC, PE, allophycocyanin (APC), APC-Cy7, and Pacific Blue (PB). The diagnostic panel comprised the following 3 tubes. Tube 1 contained anti-CD7-FITC, anti-CD20-PE-Cy5, anti-CD23, anti-CD19-AmCyan, and anti-CD19-CD56-APC, anti-CD8-APC-Cy7, anti-CD3-PB, and anti-CD4-AmCyan. CD3 and CD19 were moved to the Horizon Blue (PB). The diagnostic panel comprised the following 3 tubes. Tube 1 contained anti-CD19-PE-Cy5, anti-CD5-PerCP-Cy5, anti-CD3-PB, and anti-CD19-AmCyan. Tube 2 contained anti-FMC7-FITC, anti-CD10-FITC, anti-CD11c-PE, and anti-CD20-APC-Cy7, anti-CD5-PerCP-Cy5, anti-CD3-PB, and anti-CD19-AmCyan. Tube 3 contained anti-CD57-FITC, anti-CD23-APC, anti-CD25-APC-Cy7, anti-CD3-PB, and anti-CD4-AmCyan. CD3 and CD19 were moved to the Horizon and AmCyan channels owing to poor quality of the data in the middle of the data collection in cohort B. In addition, tube 3 was changed to contain anti-CD3-FITC, anti-CD7-PE, anti-CD5-PerCP-Cy5, anti-CD2-PE-Cy7, anti-CD56-APC, anti-CD8-APC-Cy7, anti-CD3-PB, and anti-CD4-AmCyan. All antibodies were obtained from Becton Dickinson (Franklin Lakes, NJ).

Cell Preparation

Patient lymph node biopsy specimens from a surgical procedure performed the same day or the day before were stored at 4°C on saline-soaked Telfa gauze pads until receipt in the laboratory. Single-cell suspensions were then generated by disaggregating a representative portion of fresh tissue material in phosphate-buffered saline with 1 mmol/L EDTA (PBS-EDTA). Cell concentration and viability were assessed by manual counting with trypan blue. A total of 0.5 to 1.5 × 10⁶ live cells were stained with the appropriate antibody combinations (see the “Monoclonal Antibodies” section) for 30 minutes at 4°C. Cells were then fixed and any contaminating RBCs lysed by treatment with 250 to 500 μL Opti-Lyse C (Beckman Coulter) or FACS Lysing Solution (Becton Dickinson) for 10 minutes at room temperature. Cells were then washed once with PBS-EDTA and
resuspended in the same solution before flow data acquisition. The elapsed time from tissue disaggregation to cell fixation after staining was approximately 1 hour.

**Flow Cytometry (FCM)**

**Cohort A**
Quantitative fluorescence analysis was performed using a Beckman Coulter Cytorics FC500 equipped with a single 488-nm argon laser source. FITC/PE/PC-Cy5 emission was collected in FL1/2/4 channels using 525-, 575-, and 675-nm bandpass filters, respectively. Daily instrument calibration was performed using Flow-Set/Flow-Check beads (Beckman Coulter). We noted that the voltage settings of the cytometer were changed significantly twice between 1997 and 2007; however, within the 3 time windows (1997-2002 [February], 2002 [March] to 2004 [November], 2004 [December] to 2007), these settings remained constant.

**Cohort B**
Quantitative fluorescence analysis was performed using a Becton Dickinson FACScanto II equipped with 3 lasers, a blue (488-nm, air-cooled, 20-mW solid state), a red (633-nm, 17-mW HeNe), and a violet (405-nm, 30-mW solid state) laser. FITC, PE, APC, APC-Cy7, and PB emissions were collected using 530/30- and 585/42-, 660/20-, 780/60-, and 450/50-nm bandpass filters, respectively. Daily instrument calibration was performed using BD Cytometer Setup and Tracking Beads (catalog No. 642412, Becton Dickinson).

**Immunohistochemical Analysis**
The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer’s protocol (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit, Chemicon International, Billerica, MA) on formalin-fixed, paraffin-embedded tissue (FFPET) in 95 cases of cohort A to determine the percentage of cells undergoing apoptosis.

**Gene Expression Analysis**

**Gene Expression Analysis and Determination of DLBCL Subtypes**
A total of 49 cases from cohort A had sufficient tissue available at diagnosis that a portion of the biopsy was frozen in liquid nitrogen and stored at –80°C, whereas the remaining tissue was used for FCM studies. A total of 200 μm of this fresh frozen tissue was sectioned in a cryostat, and total RNA was extracted using the ALLPREPkit (QIAGEN, Valencia, CA). Total RNA was reverse transcribed (1 cycle) and hybridized to U133-2 Plus arrays according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA).

Gene of origin was calculated using model scores for activated B-cell type and GCB type derived from the 100 gene model described by Dave et al. and the Bayesian formula described by Wright et al. A subset of 81 cases from cohort A had FFPET available for staining for BCL6 protein, MUM1, and CD10. Cell of origin was determined as GCB and non-GCB according to the Hans criteria.

**Data Analysis**

**FCM Data Analysis**
We first developed and used an automated FCM data analysis pipeline for analyzing FCM data for the 2002-2004 period for cohort A. The FCM data analysis pipeline automatically identifies (gates) all existing cell populations within an FCM file of a specific tube using the flowClust Bioconductor package. It then groups the cases according to the similarity of their FCM data (based on the percentages of cells within the identified gates) using a hierarchical clustering algorithm. Similar to the heat maps that can be generated to represent expression of genes across a number of comparable samples, we generated a heat map representing the percentages of cells within each of the identified cell populations for all cases. Details of the automated data analysis pipeline can be found in the supplementary material posted at www.ajcp.com. The significant changes in the mean fluorescence intensity of cell populations between different periods impeded us from including all samples in automated analysis. Therefore, to verify results, FCM data from all periods were manually gated using the flowCore Bioconductor package.

**Gene Expression Data Analysis**
The expression intensities for all genes/probe sets were estimated by using robust multiaarray average, with probe-level quantile normalization, as implemented in the R/Bioconductor package. Differential expression analysis was performed by using the Limma Bioconductor package. A gene was called differentially expressed if its associated q value was smaller than .05. The q value represents the P value computed by using the Limma moderated t statistic that has been adjusted for multiple testing using the method by Smyth and Storey and Tibshirani. The lists of upregulated genes in each of the groups were tested to see whether they had any associations with gene ontology (GO) terms and transcription factor binding sites. In addition to pathway analysis using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA), we used the global test to determine whether the global expression patterns of specific pathways had any associations with the identified patient groups. Global test allows the unit of analysis to be shifted from individual genes to groups of genes that represent specific pathways. In general, all statistical tests were declared significant if the q value was smaller than .05.
Statistical Analysis

Univariate survival analysis was performed using the log-rank test and Kaplan-Meier method. Overall survival (OS) was calculated from the date of diagnosis to the date of death from any cause or last follow-up alive (censored). Progression-free survival (PFS) was calculated from the date of diagnosis to the date of first progression after initiation of treatment, death from any cause, or the date of last follow-up without evidence of progression (censored). The Cox proportional hazard model was used to determine the relationship between survival and the known covariates in this study using SPSS software version 11.0 (SPSS, Chicago, IL).

Results

FCM Data Analysis

FCM data for the 57 cases in cohort A diagnosed during the 2002-2004 period were analyzed using the automated FCM data analysis pipeline. Figure 1A shows the resulting heat map of the automated analysis performed on the data for the CD5-CD19-CD3 tube (tube 4) suggesting that our automated algorithm identified 7 distinct cell populations within the CD5-CD19-CD3 tube. The dendrogram at the top in Figure 1A shows at least 3 groups of DLBCL cases (groups 1, 2, and 3 in Figure 1A) with similar FCM features. Survival analysis of these 3 groups revealed that patients clustered in group 2 had significantly inferior OS compared with the other groups (groups 1 and 3 combined; \( P = .04 \)).

Figure 1B. The defining feature of the poor outcome group (group 2) was “cell population 1” (Pearson correlation coefficient, 0.7; \( P = 9e^{-10} \)). Cases in this group had a significantly higher percentage of cells (>35%) that were characterized as being CD19+/CD3– and having a high SSC parameter, which we interpret to represent B cells with high nuclear and/or cytoplasmic complexity (hereafter referred to as high SSC CD19+ B cells). Figure 1C and 1D show pooled data for 57 samples from the 2002-2004 period and depict cell population 1 (black contour lines) superimposed over all cell populations (pseudocolor density plot).

Figure 1A, Heat map representing unsupervised hierarchical clustering of flow data. Rows in the heat map show the identified cell populations in the flow cytometry data, columns represent each patient sample, and each element of the heat map shows the percentages of cells in each of the identified cell populations. B, Overall survival of group 2 compared with all other patients. C and D, Pooled data for 57 samples from the 2002-2004 period. Cell population 1 is depicted as a contour plot (black lines) superimposed on all cell populations (depicted as a pseudocolor density plot). The lower boundary of the high side scatter (SSC) gate in the SSC dimension was defined by the corresponding upper extent of the CD19– cell population (predominantly CD3+ T cells).
Since the most prominent cell population that contributed to patient clustering was cell population 1, we hypothesized that patients from the other periods (ie, 1997-2002, n = 98; 2004-2007, n = 74) with more than 35% high SSC CD19+ B cells should have inferior survival compared with the rest of the patients. To test this hypothesis, the data for all 229 cases (including 2002-2004 cases) were manually gated to identify the percentage of high SSC CD19+ B cells. The lower boundary of the high SSC gate was defined by the upper extent of the CD19− cell population (predominantly CD3+ T cells; Figure 1C). Results of the survival analysis for the 1997-2002 and 2004-2007 periods showed that 49 (28.5%) of 172 cases had biopsy specimens containing more than 35% high SSC B cells. (Note that the cutoff threshold of 35% was determined based on the automated data analysis results.) These patients had significantly inferior OS and PFS (P = .006 and P = .04, respectively).

When all 229 cases were considered, 71 (31.0%) had biopsy specimens containing more than 35% high SSC CD19+ B cells. The high SSC profile by FCM analysis was associated with significantly inferior OS and PFS (P < .001 and P = .01, respectively) [Figure 2A] and [Figure 2B]. With a median follow-up of 5.42 years for living patients, the median OS for the high SSC group was 5.17 years; the

![Figure 2](image-url) Overall and progression-free survival of patients with diffuse large B-cell lymphoma according to side scatter (SSC) profile and treatment regimen. **A**, Overall survival (all patients). **B**, Progression-free survival (all patients). **C**, Overall survival for patients treated with rituximab and cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP). **D**, Overall survival for patients treated with CHOP-like therapy.
low SSC group did not reach median overall survival. It is
interesting that when OS curves were analyzed according
to the treatment regimen, the high SSC profile predicted
significantly inferior survival only in R-CHOP-treated
patients (n = 158; P = .001), whereas the high SSC profile in
CHOP-only treated patients trended toward inferior survival
but failed to reach statistical significance (n = 71; P = .15)
Figure 2C and Figure 2D.

It should be noted that our ability to detect more than
35% high SSC B cells is dependent on a number of factors,
including representative tissue sampling, tumor cell content
(vs background T cells, polytropic B cells, histiocytes, stromal
cells), differential viability of malignant B cells during speci-
men transport, and differential fragility of malignant B cells
during tissue processing and flow acquisition. As such, it
is possible these technical limitations precluded detection of
additional cases that might otherwise have been categorized
with the more than 35% high SSC B cell group.

To gain further insight into the extent to which cell viabil-
ity may potentially limit our analysis, we examined whether
there might be any evidence for consistent bias in sample
viability between the high and low SSC groups. Cell viability
in each sample was estimated by manual light scatter gating
where events with SSC> forward scatter (FSC) were deemed
nonviable. The average cell viability was nearly identical
between the 2 groups (74% ± 20% for the low SSC group vs
75% ± 20% for the high SSC group). In addition, Cox regres-

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<th>Variable</th>
<th>Low SCC (n = 158)</th>
<th>High SCC (n = 71)</th>
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<tr>
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<tr>
<td>Age &gt;60 y</td>
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<td>38/69 (55)</td>
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<td>29/68 (43)</td>
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<td>29/149 (17.9)</td>
<td>15/66 (23)</td>
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<td>0-2</td>
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<td>32/64 (50)</td>
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<td>3-5</td>
<td>60/137 (43.8)</td>
<td>32/64 (50)</td>
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<td>Site of biopsy</td>
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<tr>
<td>Nodal</td>
<td>129 (81.6)</td>
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<tr>
<td>Extranodal</td>
<td>29 (18.4)</td>
<td>17 (24)</td>
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<tr>
<td>Positive</td>
<td>103 (65.2)</td>
<td>55 (77)</td>
</tr>
<tr>
<td>Negative</td>
<td>25 (15.8)</td>
<td>10 (14)</td>
</tr>
<tr>
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<td>6 (8)</td>
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<td>Non-GCB</td>
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</tr>
<tr>
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<td>32 (45)</td>
</tr>
<tr>
<td>Treatment</td>
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<td>CHOP-like</td>
<td>46 (29.1)</td>
<td>25 (35)</td>
</tr>
<tr>
<td>R-CHOP</td>
<td>112 (70.1)</td>
<td>46 (65)</td>
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CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; ENS, extranodal site; GCB, germinal center B-cell type; LDH, lactate dehydrogenase; NA, not available; PS, Eastern Cooperative Oncology Group (ECOG) performance status; R-CHOP, rituximab with CHOP; SSC, side scatter; ULN, upper limit of normal.

* Data are given as number (percentage) or number/total (percentage). For some clinical variables (ie, stage, LDH, PS, and ENS), data were not available for all cases in the cohort.
To further investigate whether the high SSC B-cell population had any association with other markers, tubes 1 and 2 of the 8-color FCM data for cohort B were gated to identify high SSC and low SSC CD19+ B cells. CD11c was the only marker that had a significantly different median fluorescent intensity between the high SSC and low SSC B-cell populations (MFI\textsubscript{low-SSC B cells} = 159.0, MFI\textsubscript{high-SSC B cells} = 384.0; \( P = .017 \)). Furthermore, 36% of the cases (12/33) had more than 35% high SSC B cells, which is close to the 31% prevalence seen in cohort A.

Immunohistochemical Results

Irregular nuclear contour is a potential contributor to SSC and is also a feature of cells undergoing apoptosis. However, we did not note any obvious morphologic features of apoptosis in FFPET of the same corresponding cases by TUNEL (data not shown). Figure 3 shows representative dot plots of FCM data and corresponding H&E-stained slides of 2 representative cases with low and high percentages of cells within the high SSC gate, differences that would not be detected by H&E staining.

Gene Expression Analysis

There were considerable variations in gene expression data within the high and low SSC groups. Cell of origin seemed to be a major source, and, therefore, the variations due to the cell of origin were accounted for using the Limma Bioconductor package. Of the 393 differentially expressed genes between the low SSC and high SSC groups, there were 118 genes up-regulated in the low SSC group and 275 genes up-regulated in the high SSC group. These gene lists were tested for statistically significant associations with GO terms, transcription factor binding sites, and pathways. While some of the up-regulated genes in the low SSC gene list associated with intracellular signaling cascade, cell cycle and cell division were among the top overrepresented GO terms associated with the high SSC gene list (see supplementary tables posted at www.ajcp.com). The low SSC gene list associated with the interferon response factor, and the high SSC gene list associated with ETV4, NRF2, ELK1, and SAP1 transcription factors. Many of the genes regulated by NRF2 are involved in the cell cycle and oxidative stress response, and ELK1 and SAP1 interact with the serum response factor to mediate growth factor stimulation of the c-Fos promoter (see supplementary tables posted at www.ajcp.com). Global test results indicated that the low SSC group had higher expression in a number of pathways, including pathways related to cell cycle arrest, angiogenesis, and vascular endothelial growth factor signaling, immune signaling (tumor necrosis factor and interferon \( \gamma \)), T-cell and natural killer cell signaling, and AKT signaling pathway. The high SSC group showed higher expression in pathways related to cell cycle progression, ATM
pathway, BRCA-associated pathways, and p53 pathways (see supplementary material posted at www.ajcp.com). Pathway analysis (Ingenuity Pathway Analysis software) of the highly expressed genes for the low SSC group suggested that cell-mediated immune response and immune cell trafficking have a role in the low SSC group.

Discussion

We have described an automated method for analysis of high-complexity FCM data. This approach facilitates FCM data analysis and biomarker discovery in a high-throughput manner. By using this approach, we identified a group of DLBCL samples defined by high SSC B cells that correlated significantly with inferior survival. Analysis of an independent set of patient samples using a manual gating strategy for high SSC B cells confirmed the correlation with inferior survival and, thus, validated the usefulness of the automated approach. Given the complexity of multidimensional FCM data, we suspect that this correlation would have been difficult to discover using the traditional approach of manual gating and visualization of the data in 2-dimensional bivariate plots. We suggest that our automated approach holds potential for the discovery of novel features that may prove useful in further pathologic subclassification of known disease entities and in providing clinically relevant prognostic information.

The biologic underpinning for the high SSC feature we have identified remains uncertain. We addressed the possibility that high SSC could be a reflection of an apoptotic fraction among malignant cells by TUNEL staining of corresponding FFPET sections; however, we failed to find a significant association between these parameters. An alternative, albeit speculative, hypothesis we have considered is that the high SSC profile may be acquired as a consequence of mechanical disaggregation required to obtain single-cell suspensions before FCM analysis. This scenario would imply that perhaps acute disruption of cell-cell interactions between tumor and stroma leads to loss of a critical survival signal that thus results in increased apoptosis. To be tenable, this hypothesis would further require that more aggressive tumors bear an inherently greater apoptotic stress load that is balanced by extrinsically derived survival signals. Indeed, stromal support in DLBCL has recently been shown to be associated with an inferior survival.

Lenz et al. introduced blood vessel density and low macrophage infiltration were to be associated with clinical outcome in which high tumor blood vessel density and low macrophage infiltration were associated with an inferior survival. Lenz et al. introduced 2 gene set signatures referred to as stromal-1 and stromal-2. The prognostically favorable stromal-1 signature reflected extracellular matrix deposition and histiocytic or macrophage infiltration. By contrast, the prognostically unfavorable stromal-2 signature reflected endothelial cell biology and tumor blood vessel density. Of note, the low SSC group showed increased expression for the stromal-1 and stromal-2 genes; however, there was considerable variation in the samples, and none of the differences in expression were statistically significant for the stromal gene sets. Therefore further studies are needed to explore the underlying biologic basis for the high SSC flow phenotype.

This study suggests that high SSC among malignant B cells may serve as a useful biomarker to identify DLBCL patients at a high risk for relapse after R-CHOP. This is of particular interest because this biomarker is readily available in most clinical laboratories without significant alteration to existing routine diagnostic strategies or incurring additional costs. In routine practice, clinical DLBCL specimens may exhibit wide variation in sample viability, and the high SSC B-cell feature may not be as informative in samples with low viability. Further validation studies will be required to determine if this novel biomarker can be applied broadly and across various practice settings.

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Bashashati et al / High SSC and Inferior Survival in DLBCL