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ORIGINAL ARTICLE

CD37 and CD44 evaluation by flow cytometry: can these markers improve B cell lymphoma characterization?

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Abstract

Purpose Diffuse large B cell lymphoma (DLBCL) is a heterogenous entity with many prognostic ancillary tests that are based on immunohistochemistry. These include cell-of-origin determination by the Hans criteria and "double expresser" characterization. Additionally, *MYC* fluorescence in situ hybridization is required to distinguish DLBCL from its morphologically similar counterpart, "double hit lymphoma" or high-grade B cell lymphoma (HGBCL), a "Burkitt-like" lymphoma with a worse prognosis than DLBCL. All such ancillary tests require judicious triage of oftentimes limited specimens. Loss of expression of CD37 has been associated with worse outcomes in DLBCL. Absence of CD44 is more commonly seen in Burkitt lymphoma than in DLBCL.

Methods Very few lymphoma studies have evaluated CD37 or CD44 expression by flow cytometric analysis, which permits a more quantitative assessment. To evaluate the utility of these two markers, we retrospectively reviewed seventy-three B cell non-Hodgkin lymphoma specimens in which expression of CD37 and CD44 was evaluated by flow cytometry. Median fluorescence intensity ratio (MFIR) was calculated by dividing the median fluorescence intensity of the lymphoma population by that of background non-B mononuclear cells.

Results In lymphomas with DLBCL-like morphology, loss of CD44 expression was associated with *MYC* rearrangement (MFIR rearranged 0.3082 vs intact 1.09, p=0.003). CD44 loss in DLBCL was also associated with germinal center B cell-like (GCB) cell-of-origin (MFIR GCB 0.4139 vs non-GCB 1.499, p=0.036) as has been seen in other studies. In DLBCL, loss of CD37 expression was not associated with cell-of-origin, double expresser status, or *MYC* rearrangement.

Conclusions While our findings require confirmation in a larger case series, decreased CD44 expression by flow cytometry in an otherwise limited biopsy with DLBCL-like features may provide the impetus for FISH testing and ultimate classification.

Keywords Diffuse large B cell lymphoma · CD44 · CD37 · *MYC* rearrangement · flow cytometry

MeSH indexing Lymphoma Large B cell Diffuse; Flow cytometry Proto-oncogene proteins C-myc.

Introduction

Diffuse large B cell lymphoma (DLBCL) is the most common B cell non-Hodgkin lymphoma in the USA [1]. Since the establishment of cyclophosphamide, hydroxydaunomycin, oncovin, prednisone and rituximab (R-CHOP) therapy, there has been good overall survival and failure-free survival rates (46% and 41% respectively) [1]. However, DLBCL is a heterogeneous entity, and subsets of patients do poorly.

A variety of prognostic factors related to tumor pathobiology have been identified. Cell-of-origin determination using the Hans immunohistochemical algorithm can categorize DLBCL into the favorable, germinal center B cell-like (GCB) or the unfavorable, non-germinal center B cell-like (non-GCB) [1]. Co-expression of C-Myc and Bcl2 proteins by immunohistochemistry, the so-called double expresser (DE) immunophenotype, is seen in 20–35% of DLBCLs and predicts poorer 5-year overall survival [2, 3]. Additionally, studies have shown that gains of the *MYC* gene, as identified by fluorescence in situ hybridization (FISH), may be associated with a poor prognosis [4, 5].

A separately recognized entity with DLBCL-like histomorphology is high-grade B cell lymphoma with *MYC*

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and *BCL2* and/or *BCL6* rearrangements (HGBCL), colloquially known as "double-hit" or "triple-hit" lymphoma, which represents between 1 and 12% lymphomas with this histomorphology [6]. Despite having similar morphology to DLBCL, HGBCL is a highly aggressive B cell non-Hodgkin lymphoma defined by translocation of *MYC* with co-occurring translocation of either *BCL2* or *BCL6*. These chromosomal alterations are typically identified by FISH. HGBCL has a worse prognosis compared to DLBLC, with median survivals of 4.5–18.5 months [1]. Some studies have shown that HGBCL is more than twice as likely to be GCB by cell-of-origin [3] while DE-type DLBCL is more likely to be non-GCB [2]. Interestingly, there is a small subset of follicular lymphomas (FL) that, while low to intermediate in histologic grade, may be "double-hit" by cytogenetics [1, 7].

FISH for *MYC* and *BCL6* or *BCL2* is commonly used in practice to identify HGBCL. However, since regular use of FISH may not be cost effective, a variety of immunophenotypic and genomic markers have been evaluated to better subclassify lymphomas with DLBCL-like morphology [3, 6, 8]. One immunophenotypic marker is CD37, a cell surface glycoprotein which is a member of the tetraspanin superfamily and is complexed with integrins and other tetraspanin proteins [9]. It is present on both normal and malignant B cells. Zijun et al. demonstrated that loss of CD37 by immunohistochemistry was associated with poor response to R-CHOP [9]. We hypothesized that the absence of CD37 may thus be associated with other abnormalities predictive of a poor outcome. CD37 may also be a target for chimeric antigen receptor T-cell therapy [10, 11].

CD44 is another novel marker. It is a single-span transmembrane glycoprotein that plays roles in cell–cell interactions and cell migration [12]. Schniederjan et al. and Attarbaschi et al. each described CD44 loss by flow cytometry in Burkitt lymphoma which was not seen in DLBCL [13, 14]. Rodig et al. demonstrated that *MYC*-rearranged Burkitt lymphoma has relatively lower levels of CD44 by immunohistochemistry compared with *MYC*-intact DLBCL [15]. Diminished CD44 expression may correlate with *MYC*-rearrangement in other non-Hodgkin lymphomas.

To our knowledge, no authors have explored the relationship of CD37 or CD44 expression to *MYC* status or the DE immunophenotype. Also, very few studies have evaluated CD37 or CD44 expression by flow cytometric analysis, which permits a more quantitative assessment. Depending on the clinical practice setting, histomorphology of a given lymphoma may not be available at the time of flow cytometry. The distinction between DLBCL, HGBCL and FL is not immediately apparent by flow cytometric methods alone, and identification of *MYC* gene rearrangements may alter classification. If CD37 and/or CD44 are associated with *MYC* rearrangement, flow cytometry for these markers may better identify specimens that require *MYC* analysis by cytogenetics/FISH and aid triage of a limited specimen. We compared CD37 and CD44 expression by flow cytometry across a variety of non-Hodgkin lymphoma subtypes as well as the above-described DLBCL subtypes.

Materials and methods

Flow cytometry

All samples were submitted for clinical diagnosis and pathological evaluation to our institution's affiliated hospital. All cases were previously collected for the purpose of clinical diagnosis and patient evaluation. The relevant data is archived in our department. This study was approved by our Institutional Review Board. To retrospectively identify cases, we queried the medical record for B cell non-Hodgkin lymphoma flow cytometry cases from 6/14/2017 to 3/22/2019 in which CD44 and CD37 were evaluated.

The CD44 and CD37 evaluation consisted of a single-tube immunophenotyping panel (Table 1). Fluorochromes used included fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5), allophycocyanin (APC), allophycocyanin-cyanine tandem conjugate (APC-H7), and phycoerythrin-cyanine 7 (PE-Cy7). Cells (200,000) were incubated with fluorophore-conjugated antibodies in 200 μL of Hanks balanced salt solution (Medi-atech Cellgro, Herndon, VA, USA) with 5% human AB serum (Bio-Whittaker, Walkersville, MD, USA) for 15 min on ice without light exposure. Subsequently, the cells were washed twice with PBS by centrifugation at 500×g for 5 min and resuspended in a final volume of 250 μL of PBS. Six-color flow cytometry was performed using a FACSCanto II flow cytometer (Becton Dickinson [BD], San Jose, CA, USA) equipped with a 488-nm argon laser, a 635-nm diode laser, and FACSDiva software (BD). Daily calibration of the instrument was performed using standardized CaliBRITE Beads (BD) with FACSDiva Software (BD). On-instrument fluorescence compensation was performed using settings from compensation matrices that were confirmed daily using blood mononuclear cells. A minimum of 10,000 events were acquired. Doublets were eliminated by gating on single cells based on forward scatter area and width. Live cells and mononuclear cells were then gated based on forward and side light scatter characteristics. Lymphocyte and monocyte cell populations were identified based on side scatter characteristics (Fig. 1).

The flow cytometry data analysis was performed using FCS Express Cytometry software (De Novo Software, Los Angeles, CA, USA, http://www.denovosoftware.com/site/FCSExpress.shtml, version 6). Median fluorescence intensities were determined based on gating of the malignant and non-malignant cell populations by one of two board

Table 1 Six-color immunophenotyping panel

Fluorochrome	FITC	PE	Per-CP Cy5.5	APC	APC-H7	PE-Cy7
Antibody	CD37	CD44	CD20	CD19	CD45	CD79b
Vendor	Biolegend	BD	BD	BD	BD	Biolegend
Clone	M-B371	G44-26	L27	SJ25C1	2D1	CB3-1

certified hematopathologists. Non-B mononuclear cells (presumed T cells and monocytes) were gated by expression of CD45 and side light scatter with lack of CD19 and CD20. Their relative proportion was confirmed by CD3 expression in another screening tube, if available. B-lymphoma cells were gated on by expression of CD19 and/or CD20. This gating was informed by forward light scatter as well as degree of CD19/CD20 expression as per other screening flow cytometry tubes that included kappa/lambda light chain analysis. CD79b was also evaluated on the lymphoma cells as part of this panel but is not discussed further in this manuscript.

The median fluorescence intensity ratio (MFIR) for a given marker was calculated by dividing the median fluorescence intensity of the malignant population by the median fluorescence intensity of background non-B mononuclear cells (Fig. 1a, b). In general, background non-B mononuclear cells were categorically negative for CD37 and categorically bright for CD44. This was only calculated for cases with adequate non-B mononuclear cells; cases lacking sufficient non-B mononuclear cells were excluded.

Relative intensity for CD37 and CD44 was also determined on a subjective scale (0–4, negative to brightly positive) by one of two board certified hematopathologists if background non-B mononuclear cells were sufficient.

Immunohistochemistry

Immunohistochemistry were performed using a Ventana Ultra (Roche Inc., Basel, Switzerland) steam-induced epitope retrieval platform on positively charged slides cut from formalin-fixed paraffin-embedded blocks. A DLBCL was categorized as DE if >40% of tumor cells were C-Myc positive and >50% of tumor cells were Bcl-2 positive, per WHO guidelines [16]. The Hans criteria was used for cell-of-origin classification [17].

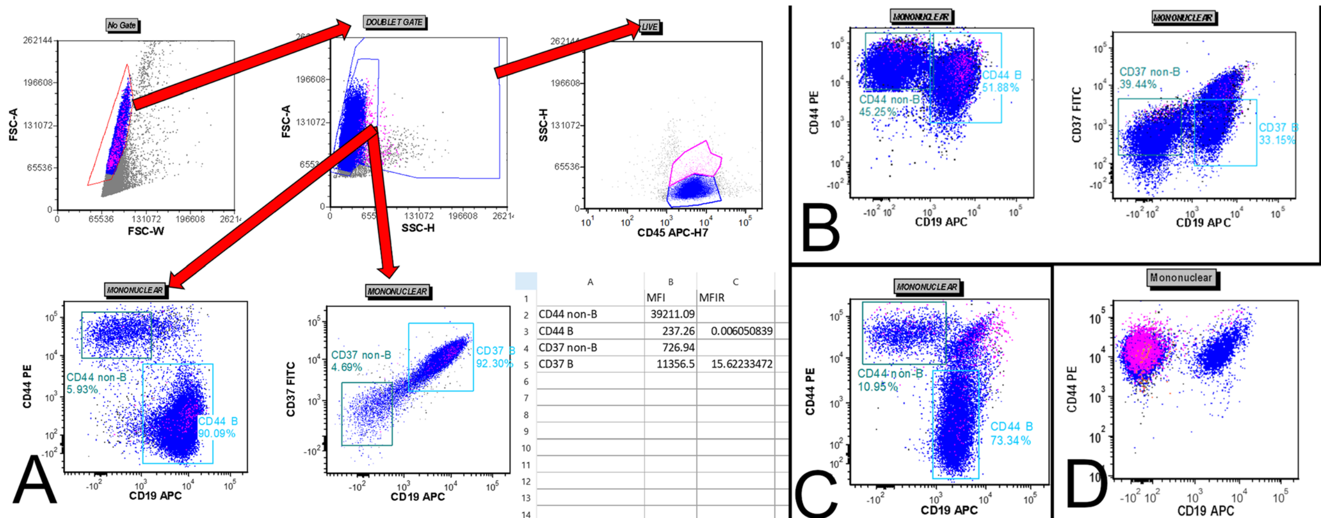

Fig. 1 **a** Flow cytometry scatter plots demonstrating gating strategy for evaluation of CD37 and CD44 expression. Forward scatter (FSC) and side scatter (SSC) axes have linear scaling while antibody fluorescence axes have biexponential scaling. Doublet events are removed based FSC area and width gating. From this gate, live events are then gated based on FSC-A and SSC properties. The low SSC events are gated separately as mononuclear cells. Using the live event gate, lymphocytes (blue) and monocytes (pink) are identified based on SSC and CD45 expression. Using the mononuclear cell gate, B cells are separated from non-B mononuclear cells (T cells and monocytes) by expression of CD19 and/or CD20. The B and non-B populations are then gated with percent of gated cells displayed. These gates are used to calculate the median fluorescence intensity of CD37 or CD44 of the given population as shown in the inset table (inset, column **b**). Median fluorescence intensity ratios are then calculated by dividing the value for B cells by non-B cells (inset, column **c**). In this example of Burkitt lymphoma, CD44 was lost with an MFIR approaching zero (0.0064), while CD37 was intact (MFIR 17.97). We show a different case (**b**), gated similarly, demonstrating near intact CD44 (MFIR 0.5117) with loss of CD37 (MFIR 2.35). Panel C shows the typical CD44 expression pattern observed in FL. Note the wide spectrum of intensity as compared to a reactive tonsil specimen, panel **d**

Chromosome studies

Chromosome spreads were G-banded by standard trypsin-Giemsa banding technique and up to twenty metaphase cells were microscopically analyzed, if the 24h and 48h unstimulated cultures provided sufficient yield. Metaphase and interphase FISH analysis for *MYC*, *BCL6*, and *BCL2* was performed using the LSI *MYC* or *BCL6* dual-color break-apart and LSI *IGH@BCL2* dual-color, dual fusion probes (Abbott Laboratories, Des Plaines, IL, USA).

Statistical methods

Distribution of variables across groups was examined by histogram plotting. Means between two categories in which both categories demonstrated a Gaussian distribution were compared with the use of a two-tailed, unequal variance (Welch's) *T*-test. Means between greater than two categories were examined using a one-way analysis of variance with the Bonferroni correction for specific contrasts during post-hoc analysis. Non-Gaussian categories were rank-ordered before applying the above means comparisons. Receiver operating curve analysis was performed assuming non-parametric distribution and a 95% confidence interval.

Statistical analysis was performed using SPSS statistics v26.0, for Windows (IBM, Armonk, NY, USA), and Microsoft Excel 2016, for Windows (Microsoft, Redmond, WA, USA). Box plots were generated for select MFIR comparisons.

Results

We queried the medical record for flow cytometry cases from 6/14/2017 to 3/22/2019, finding 81 specimens from 77 different patients. Of these, 73 specimens had adequate flow cytometry with sufficient tumor cells and background non-B mononuclear cells as determined by one of two board certified hematopathologists. Sixty-two cases had a corresponding definitive tissue diagnosis based on WHO criteria. We identified 28 cases of DLBCL, 6 cases of HGBCL, 14 cases of follicular lymphoma (FL), 2 cases of Burkitt lymphoma (BL), 5 cases of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL), 3 cases of mantle cell lymphoma (MCL), and 2 cases of marginal zone lymphoma (MZL). Additionally, one case of Burkitt-like lymphoma with 11q aberration and one case of monoclonal B lymphocytosis of undetermined significance were also identified, but these were excluded from further statistical analysis due to paucity of cases in these diagnostic groups (Table 2).

Table 2 Patient Demographics, *MYC* status and CD44, CD37 expression by diagnosis and subtype (*n* cases=60)

Diagnosis	Subtype	N cases	Gender	Age (years, mean, range)	MYC rearranged (*n*, %)	CD37 intensity score (mean, range)	CD44 intensity score (mean, range)	CD37 MFIR (mean, range)	CD44 MFIR (mean, range)
DLBCL (overall)		28	M 14, F 14	64.6, 24–94	4, 15.38%	3.36, 3–4	3.32, 1–4	23.32, 4.01–56.15	0.9988, 0.0121–4.5122
	DLBCL DE	6	M 2, F 4	74, 59–94	1, 16.7%	3.5, 3–4	3.17, 2–4	28.18, 4.75–47.73	1.4144, 0.0567–4.5122
	DLBCL non-DE	17	M 7, F 10	60.4, 24–89	1, 5.88%	3.29, 2–4	3.24, 1–4	24.33, 4.01–56.15	0.76, 0.0121–0.0567
	DLBCL GCB	12	M 6, F 6	58.6, 24–81	1, 9.1%	3.25, 2–4	3, 1–4	24.91, 6.7–56.15	0.4139, 0.0121–1.1251
	DLBCL non-GCB	11	M 3, F 8	69.7, 50–94	1, 9.1%	3.45, 2–4	3.45, 2–4	25.79, 4.01–42.55	1.499, 0.0567–4.5122
Burkitt		2	M 2, F 0	32.5, 24–41	2, 100%	3.5, 3–4	1, 1–1	27.14, 19.1–35.18	0.0069, 0.0065–0.0074
HGBCL		6	M 2, F 4	67.5, 48–82	6, 100%	3.5, 3–4	2.67, 2–4	27.97, 17.91–66.02	0.274, 0.0260–0.8328
FL (overall)		14	M 4, F 10	59.2, 18–86	3, 25%	3.29, 3–4	2.71, 2–4	20.4, 6.7–45.32	0.2797, 0.0109–0.9373
	WHO Grade I-II	6	M 1, F 5	61.3, 41–80	1, 16.7%	3.33, 3–4	2.67, 2–4	18.3, 9.15–40.02	0.2116, 0.0109–0.5193
	WHO Grade III	3	M 2, F 1	74.3, 67–86	0	3.33, 3–4	3, 3–3	24.57, 22.34–26.44	0.5536, 0.2517–0.9374
CLL		5	M 3, F 2	68.6, 49–81	0	3.2, 3–4	3.6, 2–4	22.37, 7.16–38.04	0.6503, 0.1236–1.1269
MCL		3	M 1, F 2	74.3, 72–76	0	3.33, 3–4	4, 4–4	17.91, 10.53–23.99	1.21, 0.6635–1.6361
MZL		2	M 2, F 0	68.5, 65–72	0	3, 3–3	3.5, 3–4	21.26, 7.47–35.04	0.8416, 0.8371–0.8460

Comparison of CD37 and CD44 expression by diagnosis

While there was no statistically significant difference in mean CD37 MFIR or intensity score across non-Hodgkin lymphoma diagnostic categories (p = 0.936 and 0.931, respectively), the DLBCL and FL groups did skew to lower MFIR (Fig. 2).

Mean of CD44 MFIR and intensity scores were compared across non-Hodgkin lymphoma diagnostic categories. Both CD44 MFIR and CD44 intensity score showed a statistically significant difference in means of select diagnoses (p=0.001 and 0.002, respectively). Burkitt lymphoma showed lower mean CD44 MFIR (0.0069) than DLBCL (0.9988, p=0.049) and MCL (1.21, p=0.04); however, there were only two cases in the BL diagnostic category. HGBCL had a lower mean CD44 MFIR (0.274) compared to DLBCL, CLL (0.6503), MCL (1.21) and MZL (0.8416) without achieving statistical significance (all p>0.05). FL showed lower CD44 MFIR (0.2797) versus DLBCL (p=0.038) while being similar to HGBCL. Additionally, FL demonstrated an interesting pattern of CD44 expression. CD44 expression in FL consistently demonstrated a "smear" pattern in which there was a wide range of expression within the neoplastic population (Fig. 1c). This pattern was not observed in specimens with normal, reactive germinal centers (Fig. 1d, showing reactive tonsil).

CD44 intensity scores showed a similar trend but only achieved statistical significance between BL and MCL (p=0.021). While lower, the mean CD44 intensity score for FL (2.71) was not significantly different from DLBCL (3.32, p=0.432).

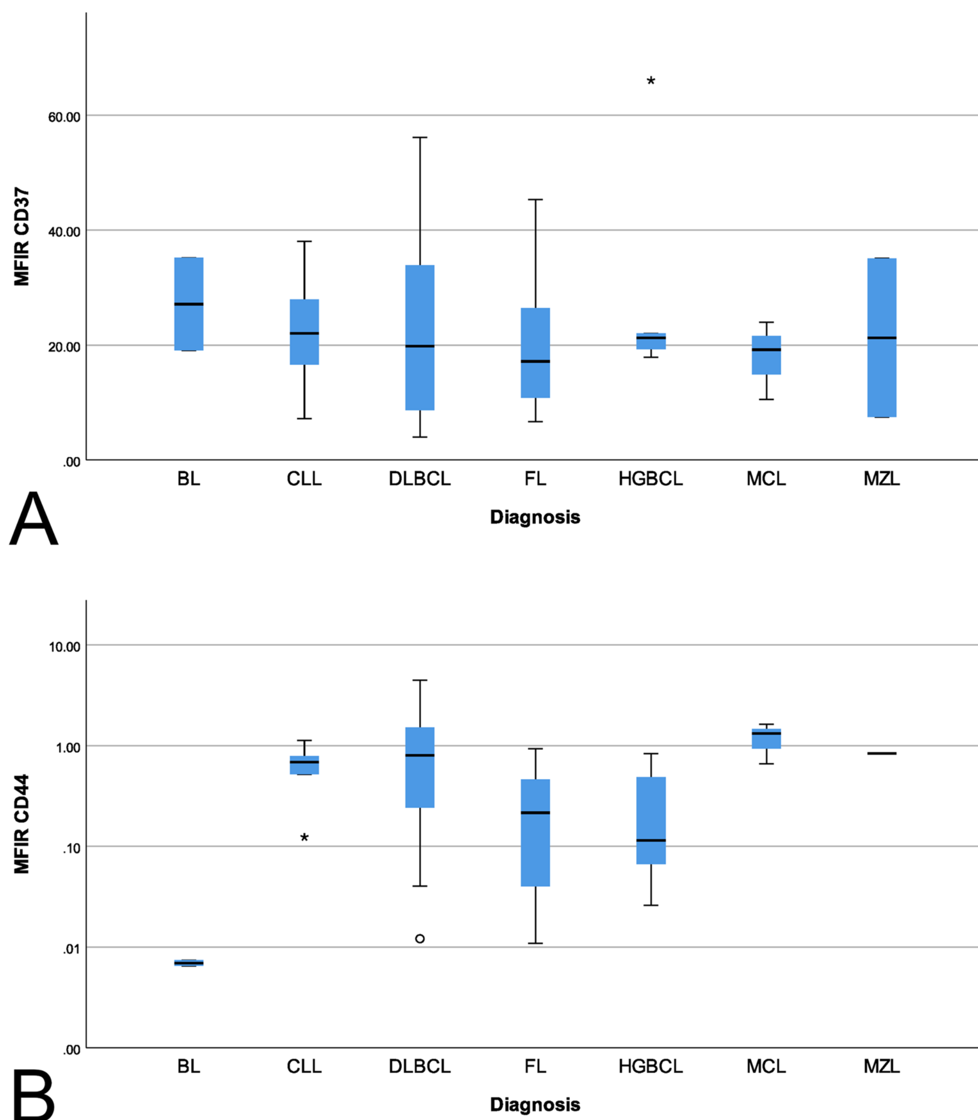

Fig. 2 Box plots of **a** CD37 MFIR and **b** CD44 MFIR grouped by diagnosis. **b** MFIR CD44 shown with logarithmic scaling. The dark horizontal line represents median, the thick area: interquartile range and the thin vertical lines: range. Points "o" and "*" represent outlier(s).

Comparison of CD37 and CD44 expression by DLBCL cell-of-origin

Twenty-three DLBCL cases had sufficient material to permit cell-of-origin classification (12 GCB, 11 non-GCB) by immunohistochemistry. CD37 MFIR (Fig. 3a, b) was nominally lower in the GCB group (24.93) versus non-GCB (25.79) and the difference was not statistically significant (p = 0.894). Similar findings were observed by CD37 intensity scoring (3.25 versus 3.45, p=0.464).

CD44 MFIR was significantly lower in the GCB group versus non-GCB (0.4139 vs 1.499, p = 0.036). CD44 intensity scoring showed a similar trend, but the difference in means did not achieve statistical significance (p=0.227).

Comparison of CD37 and CD44 expression by DLBCL DE status

Twenty-three DLBCL cases had sufficient material to permit DE subclassification by immunohistochemistry (17 non-DE, 6 DE). CD37 MFIR (Fig. 3c, d) was nominally lower in the non-DE group compared to DE (24.32 versus 28.17, p = 0.626). Similar findings were observed by CD37 intensity scoring.

CD44 MFIR was only marginally lower in the non-DE group versus DE (0.7628 vs 1.4144, p = 0.992) without achieving statistical significance, and rank of CD44 intensity showed essentially no difference between the groups.

Comparison of CD37 and CD44 expression by *MYC* status

We then examined CD37 and CD44 expression by *MYC* status when DLBCL-like histomorphology was present—and thus HGBCL could be considered at the time of initial flow cytometry diagnosis. This included 2 BL, 26 DLBCL, and 6 HGBCL cases that had concurrent *MYC* FISH studies (Table 3). CD37 MFIR (Fig. 4a) was not significantly different (*MYC* rearranged 24.08 versus *MYC* intact 26.76, p=0.665). CD44 MFIR (Fig. 4b) was lower in the *MYC* rearranged group (0.3082) compared to the *MYC* intact group (1.09), and this difference demonstrated statistical significance (p=0.003). CD44 intensity score showed a similar difference and was also statistically significant (p=0.014). Receiver operating curve analysis was performed on the CD44 MFIR dataset. It showed a sensitivity of 83.3% and specificity of 59.1% for *MYC* rearrangement when a MFIR cutoff of 0.6889 was used. CD44 intensity scoring showed

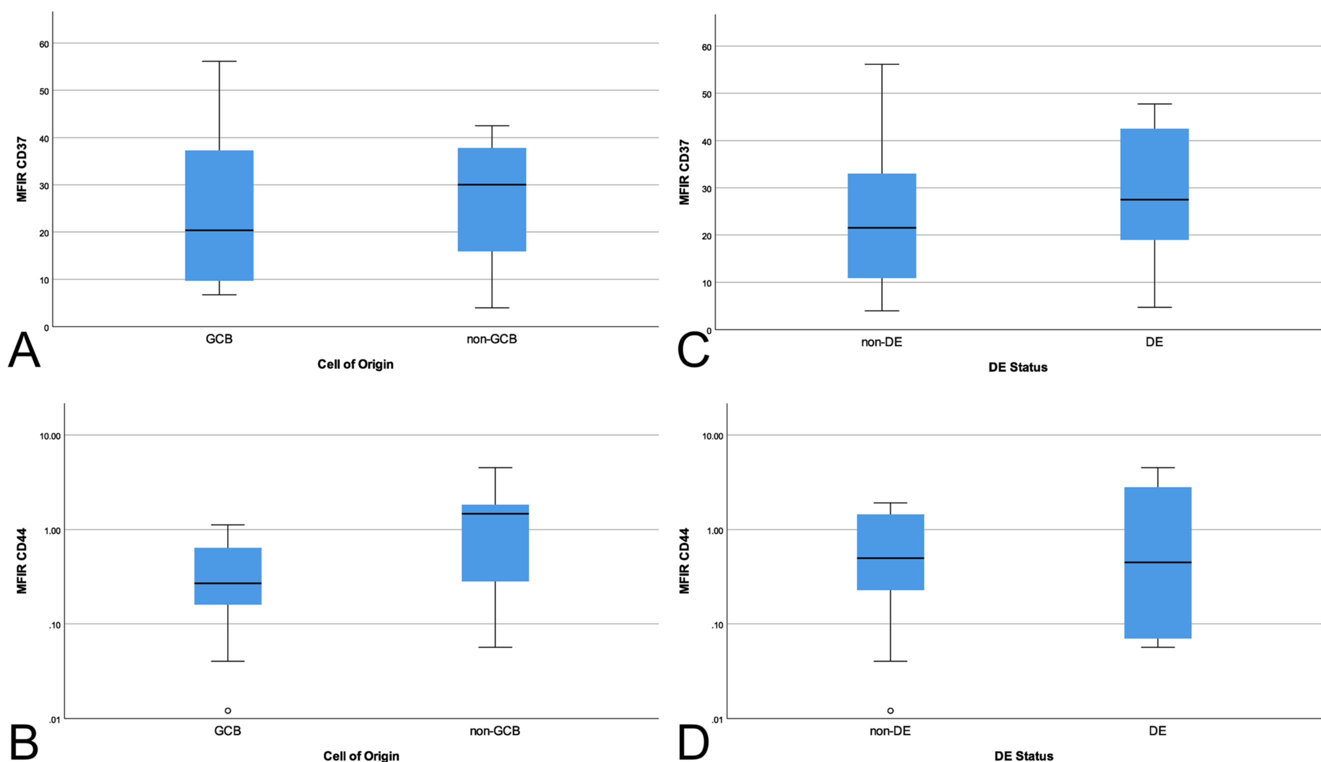

Fig. 3 Box plots of **a** CD37 MFIR and **b** CD44 MFIR for DLBCLs grouped by cell-of-origin immunohistochemical results. CD37 MFIR (**c**) and CD44 MFIR (**d**) are also examined on DLBCLs grouped by “double expresser” phenotype status. The dark horizontal line represents median, the thick area: interquartile range and the thin vertical lines: range. CD44 MFIR shown with logarithmic scaling for all comparisons

Table 3 Patient demographics, CD44, CD37 expression by *MYC* status in cases with DLBCL-like histomorphology (*n* cases=34)

Diagnosis	*N* cases	Gender	Age (years, mean, range)	CD37 intensity score (mean, range)	CD44 intensity score (mean, range)	CD37 MFIR (mean, range)	CD44 MFIR (mean, range)
MYC intact	22	M 9, F 13	67, 38–94	3.32, 2–4	3.36, 2–4	24.08, 4.01–47.74	1.09, 0.0403–4.5122
MYC rearranged	12	M 7, F 5	56.9, 24–82	3.5, 3–4	2.42, 1–4	26.76, 4.75–66.02	0.31, 0.0065–1.3689

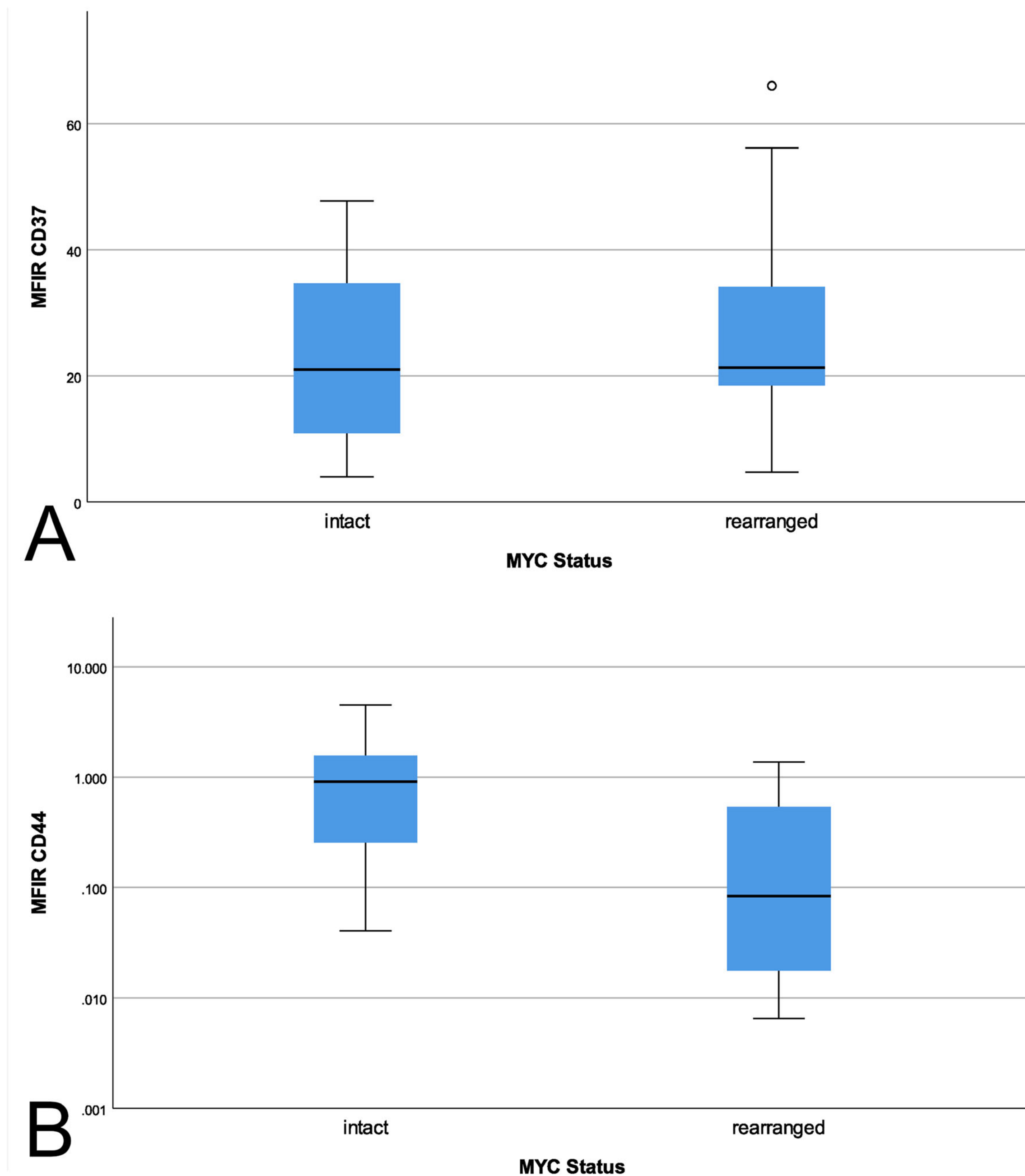

Fig. 4 Box plots of the **a** CD37 MFIR and **b** CD44 MFIR for lymphomas with DLBCL-like histomorphology in which *MYC* rearrangement evaluation was performed by FISH. The dark horizontal line represents median, the thick area: interquartile range and the thin vertical lines: range. Point "o" represents an outlier. CD44 MFIR shown with logarithmic scaling for all comparisons

similar results with 83.3% sensitivity and 50% specificity if an intensity score cutoff of 3.5 was used.

CD37 and CD44 expression in follicular lymphoma

To explore CD44 and CD37 expression in FL, MFIR was compared by *MYC* status (rearranged vs intact) as well as by histologic grade (WHO Grade I-II vs IIIA/IIIB), if available.

Of the FL cases, 9 had a concurrent histologic grade (6 WHO grade I-II and 3 WHO grade III). CD37 MFIR was lower in WHO I-II (18.3) versus WHO III (24.57). CD44 MFIR was lower in the WHO I-II group (0.2116) versus WHO III (0.5536) (see Fig. 5a, b). However, the mean differences in CD37 MFIR and CD44 MFIR did not achieve statistical significance (all $p>0.05$). A similar pattern was observed by intensity scoring.

Of the FL cases, 12 had concurrent flow cytometry and *MYC* FISH studies. Three FL cases had *MYC* gene rearrangement in addition to *t*(14; 18). CD37 MFIR was not significantly different in *MYC* rearranged compared to intact (22.49 versus 19.98, $p=0.853$). No substantial difference was noted by intensity score. The *MYC* gene rearranged FL group showed lower CD44 MFIR compared to intact (0.184 versus 0.305) but did not achieve statistical significance ($p = 0.903$) (see Fig. 5c, d). The above described "smear" pattern was present in the *MYC* rearranged FL cases as well as the *MYC* intact FL cases. Of the MYC rearranged FL cases, only one had a tissue biopsy for grading. This specimen had low-grade morphology (WHO Grade I-II) with a low Ki-67 proliferation rate (20%) by immunohistochemistry.

Discussion

Interpretation of our study's data is limited by the low overall number of cases collected, particularly those in the BL and HGBCL categories. We rated CD37 and CD44 intensity by two methods: a subjective intensity score and MFIR. The former is akin to how a practicing hematopathologist would interpret a flow cytometry study. The latter permitted more subtle quantitation of expression as compared to background. Both rating methods showed similar trends across comparisons, and both methods achieved statistical significance in many contrasts. The exceptions to this were CD44 intensity score in BL vs MCL and CD44 intensity score in GCB vs non-GCB. Intensity score, in both of these, showed a similar trend as MFIR but did not achieve statistical significance. However, the number of cases in the BL and MCL groups was very low. Overall, this provided some degree of validity to the categorical evaluation of flow cytometry employed by practicing hematopathologists regarding CD37 and CD44.

As CD44 has been used to aid in diagnosis of Burkitt lymphoma, we hypothesized that CD44 loss may be associated with *MYC* rearrangements observed in other non-Hodgkin lymphomas. Specifically, we examined CD44 loss in lymphomas with DLBCL-like histomorphology. In this group of

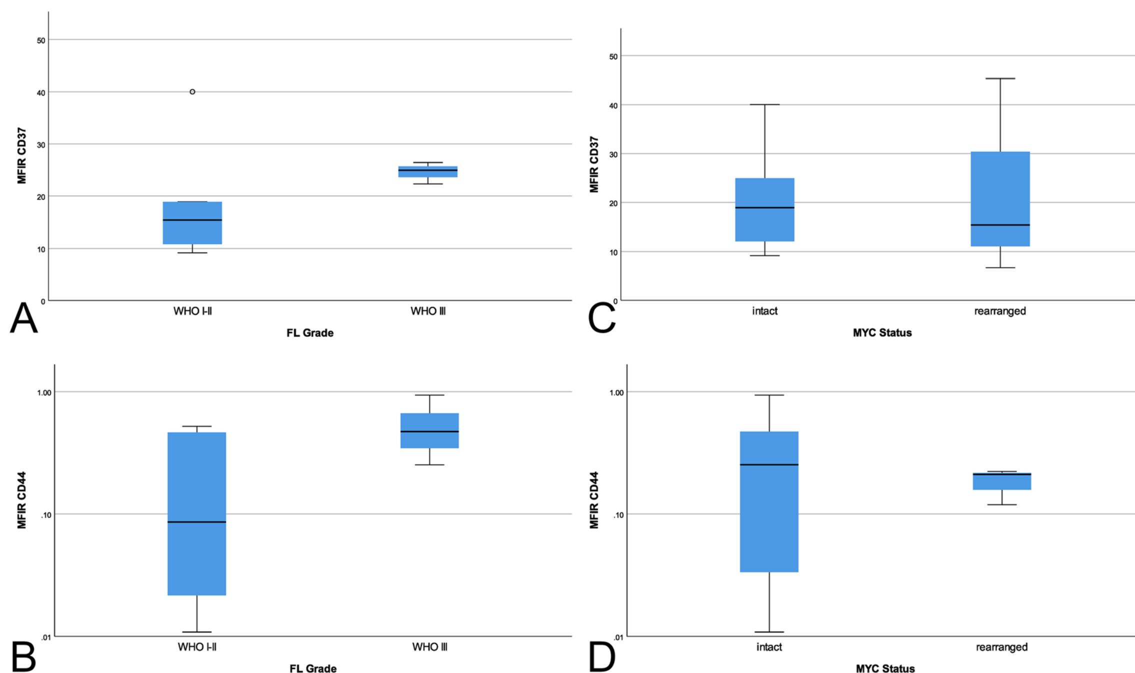

Fig. 5 Box plots of the **a** CD37 MFIR and **b** CD44 MFIR in FL grouped by histologic grade. Box plots of **c** CD37 MFIR and **d** CD44 MFIR in FL grouped my *MYC* rearrangement status also shown. The dark horizontal line represents median, the thick area: interquartile range and the thin vertical lines: range. Point "o" represents an outlier. CD44 MFIR shown with logarithmic scaling for all comparisons

lymphomas, *MYC* rearrangement was associated with CD44 loss. CD44 loss was also associated with GCB cell-of-origin. This finding is consistent with other studies that have shown HGBCL to be more likely of GCB immunophenotype by the Hans criteria [3]. Such cell-of-origin classification can mislead clinicians as HGBCLs have worse outcomes than would be predicted by being GCB. CD44 loss only correlated with *MYC* rearrangement and not overexpression of the C-Myc protein as noted in the DE grouping. Overexpression of C-Myc protein by immunohistochemistry does not require *MYC* gene rearrangement as many other genetic events could lead to increased production of C-Myc. Receiver operating curve analysis showed moderate sensitivity (83.3%) and poor specificity (59.1%) for classifying as *MYC* rearranged with a CD44 MFIR cutoff of 0.6889 selected. Regarding CD44 intensity score, a cutoff of 3.5 provided a similar sensitivity. As CD44 intensity of background normal T cells was rated as 4.0, this relatively high cutoff suggests that any loss in intensity in the lymphoma population may have a degree of sensitivity for *MYC* rearrangement. Given sufficient clinical and histologic pre-test probability, identification of CD44 loss at the time of flow cytometry could prompt a practicing pathologist to triage a limited biopsy specimen for *MYC* FISH studies. This finding could also be useful in practices that do not routinely perform FISH on all B cell non-Hodgkin lymphoma specimens.

We hypothesized that CD37, which has been shown to be lower in DLBCLs that respond poorly to R-CHOP, may be lower in other subgroups of DLBCL that also have poor prognoses. However, no significant differences in CD37 expression were noted based on cell-of-origin, double-expresser, or *MYC* rearrangement grouping in DLBCL. The difference in survival rates, shown in other studies, may be attributed to heterogeneity of the disease not accounted for in our retrospective study.

Interestingly, our study included three FL cases with *MYC* rearrangements. While there were too few cases to draw serious conclusions, a lower trend was observed for CD44 expression in the *MYC* rearranged FL cases vs the *MYC* intact. However, the "smearing" pattern was present in all cases and such a wide range of expression confounds the practical utility of CD44 analysis in FL with regard to triaging for *MYC* FISH.

Since our retrospective review included all B cell non-Hodgkin lymphoma diagnoses in which CD37 and CD44 were evaluated by flow cytometry, we were able to examine differences in expression by diagnosis. While we only had two cases of BL in our set, we saw similar loss of expression of CD44 as described by other authors [14, 15]. FL showed a unique "smearing" pattern of expression for CD44, to our knowledge, not described elsewhere in the literature (Fig. 1c). This pattern was not observed in other lymphoma subtypes in our review. Its uniqueness could be leveraged to ensure a specimen is appropriately triaged for *IGH-BCL2* rearrangement FISH, particularly if the flow cytometry data is not reviewed by the same pathologist as the surgical specimen. No significant difference was observed grouping by FL histologic grade.

The low number of cases in some diagnostic categories limited statistical evaluation in our study. However, many similar studies we have referenced above also had a relatively low number of cases. Some cases in our retrospective lacked a concurrent tissue specimen which precluded analysis that required definitive diagnosis and classification. We feel that these limitations highlighted the difficulties present in real clinical practice and emphasized the utility of CD44 evaluation for specimen triage. In fact, many cases in our retrospective originated from outside our institution as "flow only" specimens. As such, clinical follow-up information was limited. This barred thorough outcome and survival modeling. Future directions could include evaluation of a more comprehensive prognostic algorithm that includes CD37 and CD44 immunophenotyping as well as longer term follow-up of patients.

Flow cytometric analysis is a valuable tool for the diagnosis and triage of limited specimens. Decreased CD44 expression by flow cytometry in an otherwise limited biopsy with DLBCL-like features may provide the impetus for FISH testing and ultimate classification.

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