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Original contribution

Comparison of evaluation techniques, including digital image analysis, for MYC protein expression by immunohistochemical stain in aggressive B-cell lymphomas☆,☆☆

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Summary Incorporation of an MYC immunohistochemical stain in the workup of large B-cell lymphomas has become common in hematopathology practice. Evaluation of this stain can be difficult because of staining heterogeneity and can have interobserver variability, particularly when performed on the entire tumor sections. We identified 87 cases of aggressive B-cell lymphoma (34 core needle and 53 excisional biopsies) and compared the following methods of MYC immunohistochemical staining evaluation: the original pathologist's interpretation, a systematic retrospective method of evaluation by manual analysis, and a retrospective method of evaluation by digital image analysis (using scanned slides analyzed via the Aperio Nuclear algorithm). Overall, concordance among these methods was around 80% with κ statistics showing good agreement. However, nearly one-third of our cases had a percent MYC positivity in the 30% to 50% range, and for these cases, concordance among the various methods was marginal/poor. This suggests limited utility as a prognostic or predictive marker using 40% as a cutoff value. In our series, core biopsy specimens were poor predictors of *MYC* gene rearrangement, and there was no association between MYC immunohistochemical stain and *MYC* gene gain/amplification. Our retrospective digital image analysis showed strong correlation in MYC percent positivity with our retrospective manual review (correlation coefficient of 0.90) and similar concordance to pathologist interpretation as among pathologists, suggesting that digital image analysis is a viable alternative to manual determination of MYC percent positivity. Digital image analysis provides further opportunities for more sophisticated and standardized scoring systems, which may be helpful in future prognostic/predictive studies.

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1. Introduction

Rearrangement of the *MYC* gene is well described in aggressive B-cell lymphomas including Burkitt lymphoma (nearly all cases) and diffuse large B-cell lymphoma (DLBCL; 8%-14% of cases) [1]. A higher proportion of DLBCLs show MYC protein expression (detected in approximately 30% of cases) than *MYC* gene rearrangement [2,3]. Translocation of the *BCL2* gene occurs in 20% to 30% of DLBCL cases, and rearrangement of the 3q27 region involving *BCL6* is seen in up to 30% of DLBCL [1]. Cases of DLBCL or cases with morphologic features of both Burkitt lymphoma and DLBCL (referred to in the 2008 World Health Organization [WHO] classification as "B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma") and harboring a *MYC* (8q24) rearrangement in combination with a *BCL2* (18q21) and/or a *BCL6* (3q27) rearrangement have a relatively low complete response rate and short overall survival with R-CHOP or comparable therapies. These lymphomas, sometimes referred to as "double-hit" and "triple-hit" lymphomas, are separately classified according to the 2016 WHO classification as "high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements." Dual positivity by immunohistochemical (IHC) stains for MYC and BCL2 in DLBCL (double expression of MYC and BCL2 proteins) is associated with inferior survival in most studies [2-4]. Such double expression may be a prognostic marker but does not warrant separate classification according to the 2016 WHO classification.

As MYC and BCL2 protein expression may have prognostic significance and there is theoretical use for MYC IHC staining to triage which aggressive B-cell lymphomas require genetic studies to evaluate for double or triple hit, the incorporation of an MYC IHC stain has become common in hematopathology practice. After the standard validation process for a new IHC stain [5], our institution began offering the MYC IHC stain with a recommended cutoff (≥40% as positive) in early 2015. Unfortunately, IHC evaluation of this stain can have interobserver variability, particularly when performed on the entire tumor sections by a diverse group of pathologists [6]. Digital image analysis is an emerging reproducible method of quantifying positivity by IHC stains [7-9].

We studied cases of aggressive B-cell lymphoma (Burkitt lymphoma, DLBCL, and high-grade B-cell lymphoma) with the MYC IHC stain performed as part of routine clinical care. Our aims were (1) to compare multiple methods of MYC IHC staining evaluation (the original pathologist's interpretation, a systematic retrospective method of evaluation by manual analysis, and a systematic retrospective method of evaluation by digital image analysis) and (2) to assess the ability of MYC IHC staining to predict a *MYC* gene rearrangement or *MYC* gene gain/amplification.

2. Materials and methods

Our institutional review board approved this retrospective study. We used a Sunquest CoPathPlus (version 6.1.1 Sunquest Information Systems, Tucson, AZ) database search to identify cases of large B-cell lymphoma with an MYC IHC stain performed as part of routine clinical care from the time the stain was made available in March 2015 through December 31, 2016. During this period, the MYC IHC stain was not used as a triaging tool for cytogenetic analysis at our institution. The MYC IHC stain (clone Y69; dilution 1:229; Abcam, Cambridge, MA) was performed using the standard procedures of our IHC laboratory at the time. The original pathologist's interpretation of MYC staining (positive, negative, or equivocal) was obtained from pathology reports and is referred to as "original IHC interpretation" in the Results section. The results of cytogenetic studies (fluorescence in situ hybridization, or FISH), performed using standard methods for our laboratory at the time of biopsy, were recorded when performed on the same tissue sample as the IHC stain. An *MYC* break-apart probe was used (Vysis LSI MYC dual-color break apart rearrangement probe; Abbott Molecular, Abbott Park, IL) to determine *MYC* rearrangement and gain/amplification status. Gain/amplification of the *MYC* gene for the purposes of this study was defined as greater than 2 *MYC* fusion signals. We evaluated the hematoxylin-and-eosin (H&E) and MYC IHC–stained slides for sufficient tissue and quality of specimen/staining, with any cases with insufficient tissue or missing/unavailable slides excluded. Up to 10 areas on each H&E slide measuring approximately 1 × 1 mm were manually marked with a dotting pen. Areas with the best quality and most neoplasm-rich tissue were selected, whereas areas with crush artifact, fibrosis, and necrosis were avoided. The corresponding areas on the MYC-stained slides were also manually marked. Fig. 1 illustrates the methods of retrospective review.

For the retrospective review by digital image analysis, H&E and MYC IHC slides for each case were scanned at ×40 using an Aperio ScanScope XT whole-slide scanner (Leica Biosystems, Buffalo Grove, IL), including on-slide control tissue. These were converted to digital images and stored on a password-protected database using Aperio eSlide Manager (version 12.3; Leica Biosystems). The MYC-stained slides were annotated using Aperio ImageScope viewing software (version 12.3; Leica Biosystems). The ruler tool was used to measure 1 × 1 mm squares in each area previously marked by the dotting pen on the slide, then the free-hand pen tool or rectangle tool were used to annotate areas for analysis. The annotations on each MYC-stained slide were analyzed using the Aperio Nuclear algorithm (version 9.2, Leica Biosystems), with no manipulation to the algorithm. Default settings for the nuclear algorithm were used (*Nuclear Algorithm, User's Guide*; Leica Biosystems, MAN-0338, Revision 8; August 5, 2015). All data were saved individually by case, and the numbers of negative, 1+, 2+, and 3+ nuclei were specifically collated. We did not use the negative versus positive (1+ through 3+) pixel count for this study.

For the manual retrospective review, a university-based hematopathologist reviewer (pathologist 1) graded the percent of cells with positive nuclear staining for each area previously

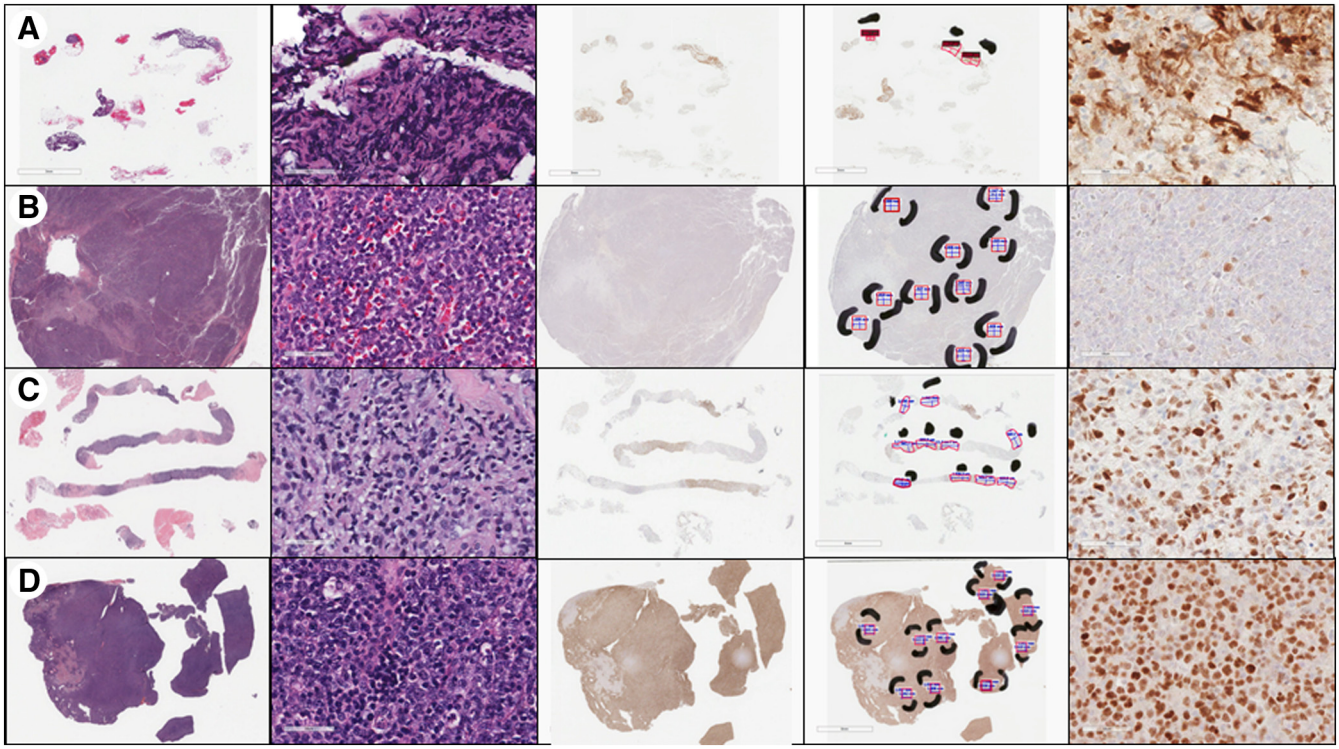

Fig. 1 Images from representative cases showing insufficient material for review (row A), negative MYC IHC staining (row B), equivocal MYC IHC staining (percent positivity in 30%-50% range; row C), and positive MYC IHC staining (row D). The first column shows whole-slide images of the H&E-stained slide. The second column shows a portion of the H&E slide at ×400 magnification in one of the selected areas of best tissue. The third column demonstrates whole-slide images of the MYC IHC slide. The fourth column shows the Aperio ImageScope annotations on the MYC IHC slide, with black marking pen indicating the areas of best-quality tissue selected as the areas to evaluate. The red boxes select tissue to analyze, whereas the text and ruler icons in blue measure the area selected, with the goal of 1 mm^2 in each area. The fifth column shows the MYC IHC slide at ×400 magnification, within one of the evaluation areas.

marked by the dotting pen on the MYC-stained slide. The percentage of nuclei with brightly positive staining was recorded as well as the percentage of nuclei with any staining (ie, dim and bright alike). An average from all marked areas (up to 10 per slide) was calculated to obtain separate percent positive staining values for dim staining and any staining. When the final value for percent positivity fell between 30% and 50% by manual retrospective review, a second university-based hematopathologist reviewer (pathologist 2) performed the same grading process. On-slide control tissue and the corresponding H&E-stained tissue slide were evaluated as needed. Pathologists 1 and 2 were blinded to the retrospective digital image analysis, which was performed by a third pathologist.

To test the association between *MYC* gene status and variables of interest, Wilcoxon rank sum tests and Fisher exact tests were performed, as appropriate. Correlation was evaluated using Spearman correlation coefficients and *t* tests. Concordance was evaluated using the κ statistic [10]. Specificities and sensitivities were calculated. All reported *P* values are 2 sided. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC) and R version 3.4.0 (http://www.R-project.org/).

3. Results

3.1. General characteristics

Our cohort consisted of 87 cases: 72 (83%) DLBCL or high-grade B-cell lymphoma (as defined by 2016 WHO classification), 12 (14%) monomorphic PTLD, and 3 (3%) Burkitt lymphoma (Fig. 2). Patients ranged in age from 3 to 93 years at the time of biopsy with an average age of 58 years. More of the biopsies were from female than male patients (50 female, 37 male). Sampled sites included the following: lymph node (n = 34), soft tissue and bone (26), oropharynx/sinonasal (7), gastrointestinal tract (4), liver (4), brain (4), gonadal (3), and 1 each from salivary gland, breast, omentum, thyroid, and lung. Thirty-four cases were core biopsies and 53 were excisional biopsies. For the manual retrospective review and retrospective review by digital image analysis, 32 samples had less than the goal of 10 areas evaluated, of these, 19 were core biopsies and 13 were excisional biopsies. For these 32 samples, an average of 5.2 areas were evaluated.

For each case, the original IHC interpretation was made as part of routine patient care by 1 of 12 different pathologists—6

fellowship-trained hematopathologists practicing at a university hospital and 6 community-based pathologists (some with hematopathology fellowship training). Most cases were originally signed out by the university-based hematopathologists (72%), with the remainder signed out by community-based pathologists. We saw no pattern differences in MYC interpretation between the university hospital and community-based pathologists (data not shown).

3.2. Distribution and correlation of percent MYC positivity

Fig. 3 illustrates the distribution of percent MYC positivity by retrospective manual review (pathologist 1) and retrospective digital image analysis for any staining and 1+ to 3+, respectively. See Supplementary Fig. S1 for the distribution of bright staining only by retrospective manual review and 2+ to 3+ by digital image analysis. It can be seen there is a wide spectrum of MYC percent positivity by both methods. There was a strong positive correlation for MYC percent positivity between manual retrospective review by pathologist 1 and retrospective review by digital image analysis, with a Spearman correlation coefficient of approximately 0.90 ($P < .01$) for all comparisons (manual any staining, manual bright staining versus digital image analysis 1+ to 3+, digital image analysis 2+ to 3+). A representative scatterplot is shown in Fig. 4. When we restricted analysis to the 22 cases with a percent MYC positivity between 30% and 50% (as determined by manual retrospective review by pathologist 1), the correlation to retrospective review by digital image analysis was reduced with a Spearman correlation coefficient of 0.438, and there was no correlation between pathologist 1 and pathologist 2 (Spearman correlation coefficient of 0.236, $P = .291$).

3.3. Concordance among methods of MYC IHC staining evaluation

The original sign-out pathologist interpreted the MYC IHC stain as positive in 41 cases (47%), negative in 37 (43%), and equivocal in 8 (9%), with an interpretation not given in 1 (1%). Using a cutoff of ≥ 40% as positive, 51 (68%) of the 75 cases deemed sufficient material for evaluation by the manual retrospective method were positive for any nuclear staining, and 30 (40%) of the 75 cases were positive for bright nuclear staining. Twelve cases were deemed insufficient material to evaluate by the manual retrospective method but were evaluated by retrospective digital image analysis. Using a cutoff of ≥ 40% as positive, 40 (46%) of the 87 cases evaluated by retrospective digital image analysis were positive when 1+ through 3+ nuclear positivity was interpreted as positive, and 12 (14%) of

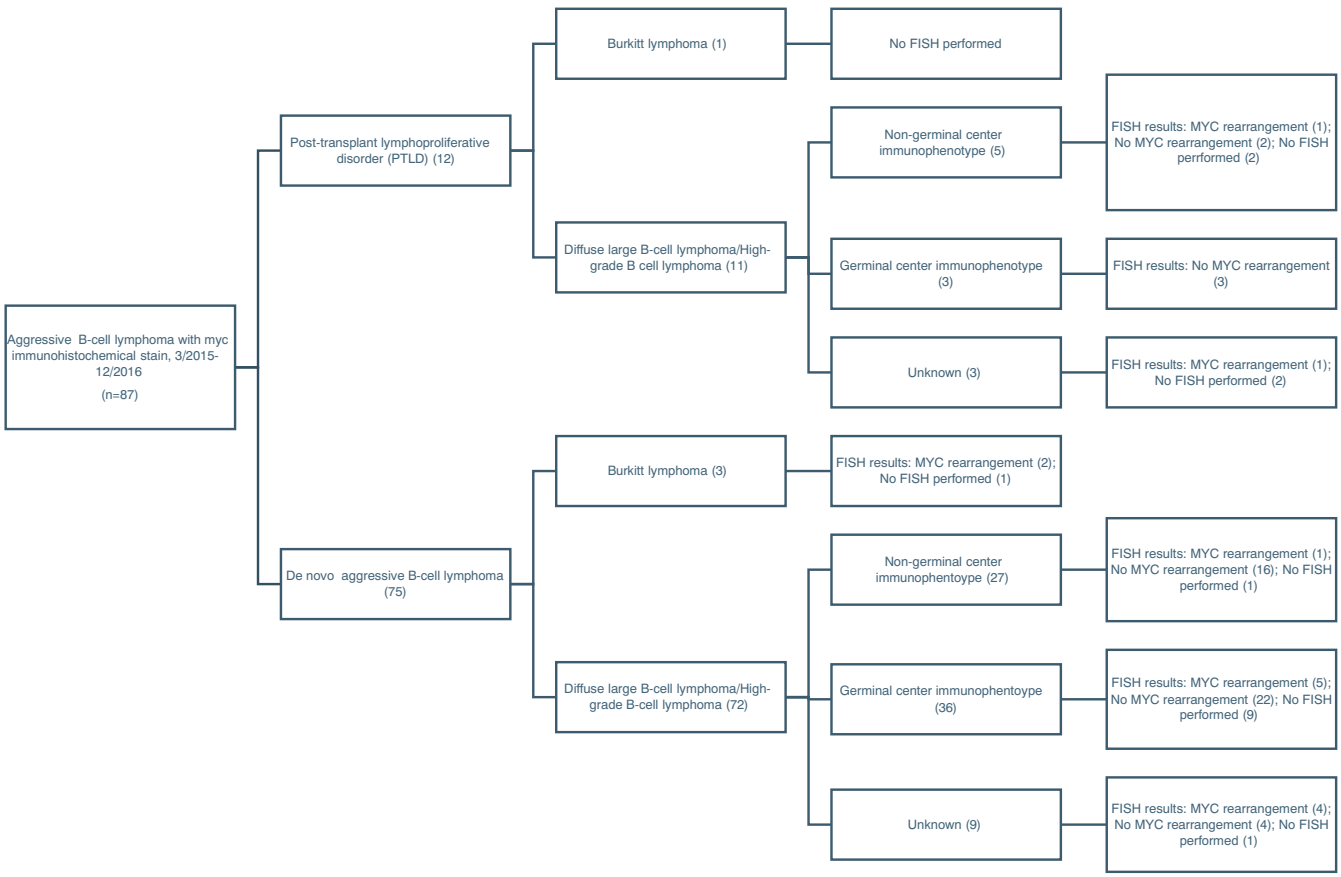

Fig. 2 Flowchart outlining the classification of aggressive B-cell lymphomas included in our study.

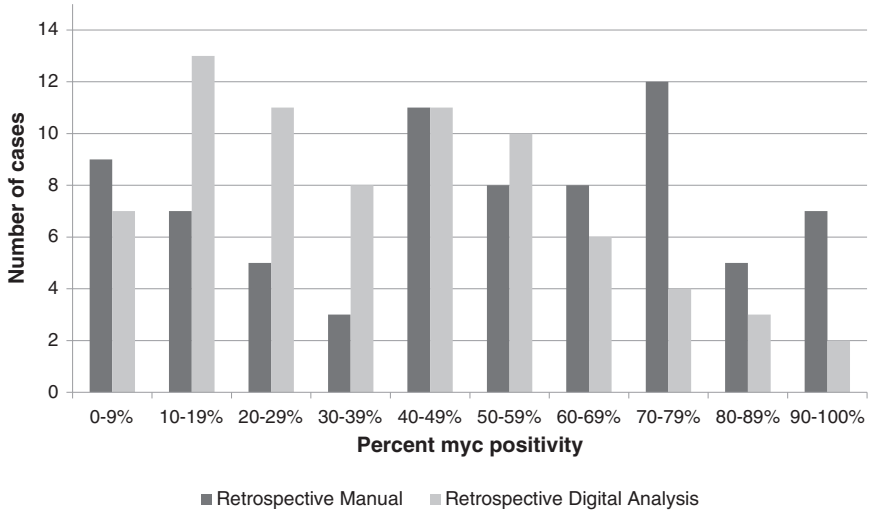

Fig. 3 Distribution of percent MYC positivity by retrospective manual review (pathologist 1) and retrospective digital image analysis. Represented in this figure are the percentages using any nuclear staining (retrospective manual) and 1+ to 3+ nuclear positivity (retrospective digital analysis). Both the manual and digital analysis methods show wide variability in percent MYC positivity, including many cases surrounding the suggested cutoff of 40%. The manual method showed a bias toward higher estimation of MYC percent positivity, whereas the digital analysis method showed a bias toward lower estimation of MYC percent positivity.

the 87 cases evaluated by retrospective digital image analysis were positive when 2+ through 3+ nuclear positivity was interpreted as positive.

Concordance values among the 3 methods of MYC IHC stain evaluation (comparing interpretation of "positive" or "negative") are shown in Table 1. There was excellent concordance between the retrospective manual review and the digital image analysis on the core biopsy specimens; there was good concordance between all other comparisons. When evaluation was limited to the subset with staining in the 30% to 50% range (all specimens), concordance values were 50%, 60%, and 45%, respectively, for original IHC interpretation to manual retrospective review, original IHC interpretation to retrospective review by digital image analysis, and retrospective manual review to retrospective review by digital image analysis. Corresponding κ scores were 0.138, 0.130, and 0.137,

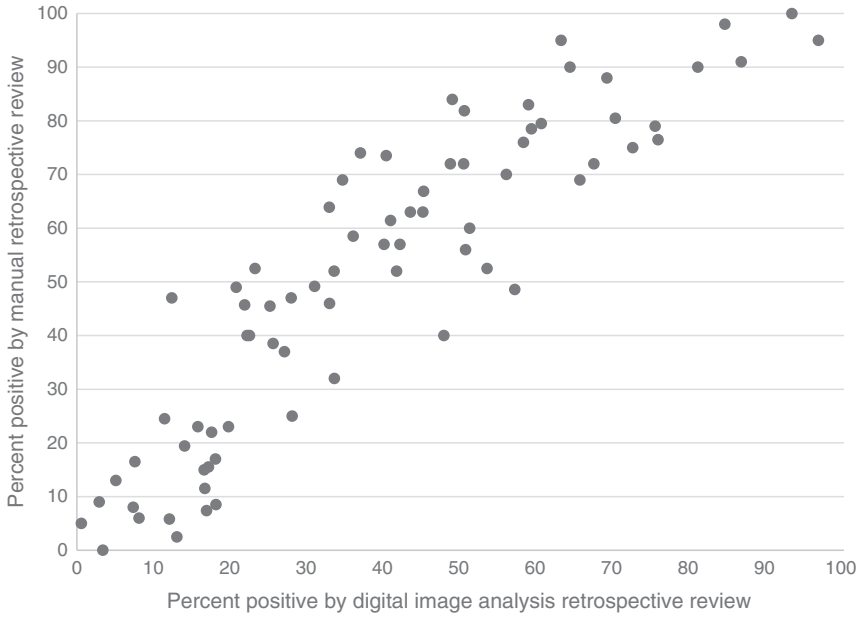

Fig. 4 Scatterplot demonstrating correlation between retrospective manual review of MYC IHC positivity and digital review by Aperio ImageScope with nuclear algorithm (Spearman correlation coefficient of 0.92).

Table 1 Concordance among methods of MYC IHC stain evaluation [a]

	Concordance (%)	Cohen κ statistic	Agreement [b]
Original IHC interpretation to manual retrospective review (n = 69)	80	0.588	Good
Original IHC interpretation to retrospective review by digital image analysis (n = 78)	81	0.616	Good
Retrospective manual review to retrospective review by digital image analysis (n = 75)	80	0.606	Good
Core biopsy only			
Original IHC interpretation to manual retrospective review (n = 23)	87	0.738	Good
Original IHC interpretation to retrospective review by digital image analysis (n = 30)	80	0.598	Good
Retrospective manual review to retrospective review by digital image analysis (n = 24)	96	0.915	Excellent
Excisional biopsy only			
Original IHC interpretation to manual retrospective review (n = 46)	76	0.513	Good
Original IHC interpretation to retrospective review by digital image analysis (n = 48)	81	0.626	Good
Retrospective manual review to retrospective review by digital image analysis (n = 51)	73	0.474	Good

[a] Concordance evaluated using any nuclear staining by manual retrospective review and 1+ through 3+ nuclear positivity by digital image analysis to determine percent MYC positivity and using a cutoff of at least 40% as positive.

[b] As defined by Le [10].

Table 2 MYC IHC staining versus *MYC* gene rearrangement status (Excisional biopsy specimens only)

	MYC gene rearrangement (n = 9) [a]	No *MYC* gene rearrangement (n = 28) [a]	*P*
Myc immunostain percent positivity, median (25%, 75%)			
Digital image analysis (1+ to 3+)	65.6 (63.1, 80.9)	29.5 (14.7, 43.4)	.001
Digital image analysis (2+ to 3+)	30.7 (19.3, 51.3)	14.0 (5.3, 23.8)	.02
Manual retrospective (any staining)	90.0 (81.9, 91.0)	48.6 (23.0, 63.9)	.0005
Manual retrospective (bright staining)	67.5 (38.1, 85.0)	25.0 (9.0, 42.8)	.009
Original pathologist's interpretation of MYC staining, n (%)			.002
Positive	9 (100)	9 (32.1)	
Negative	0 (0.0)	15 (53.6)	
Equivocal	0 (0.0)	4 (14.3)	

[a] Overall n may vary by variable.

Table 3 Sensitivity and specificity of the various methods of MYC IHC stain interpretation for *MYC* rearrangement determination

	Sensitivity		Specificity	
Original IHC interpretation				
All specimens (n = 54)	0.93		0.65	
Germinal center subtype (n = 28)	0.80		0.70	
Nongerminal center subtype (n = 16)	1.00		0.57	
Core (n = 21)	0.80		0.69	
Excision (n = 33)	1.00		0.63	
Manual retrospective review [a]	Any	Bright	Any	Bright
All specimens (n = 55)	0.92	0.62	0.40	0.69
Core (n = 19)	0.75	0.50	0.53	0.67
Excision (n = 36)	1.00	0.67	0.33	0.70
Retrospective review by digital image analysis [b]	Any	Bright	Any	Bright
All specimens (n = 61)	0.86	0.50	0.66	0.98
Core (n = 24)	0.80	0.60	0.63	1.00
Excision (n = 37)	0.89	0.44	0.68	0.96

NOTE. Of the 61 cases with FISH analysis, 6 were deemed insufficient to evaluate by retrospective manual review, and 7 were deemed equivocal by original pathologist's interpretation, accounting for varying "n."

[a] For the manual retrospective review, "any" refers to MYC IHC percent positivity coming from nuclear staining of any intensity (bright or dim) and "bright" refers to MYC IHC percent positivity coming from nuclear staining of only bright intensity.

[b] For the digital image analysis, "any" refers to MYC IHC percent positivity coming from 1+ through 3+ nuclear positivity and "bright" refers to MYC IHC percent positivity coming from 2+ through 3+ positivity.

respectively, indicating marginal/poor agreement. There was marginal/poor agreement between pathologist 1 and pathologist 2 for the subset of cases staining in the 30% to 50% range.

3.4. MYC IHC stain versus *MYC* gene status

Sixty-one cases were evaluated by FISH for *MYC* gene rearrangement on the same tissue sample as the MYC IHC stain. Fourteen (23%) of 61 tested cases had an *MYC* rearrangement by FISH analysis. A summary of MYC IHC staining (as evaluated by the various methods) compared with the presence or absence of an *MYC* gene rearrangement in excisional biopsy specimens is presented in Table 2. There was a significant association between MYC IHC staining and *MYC* gene rearrangement status for all methods when assessing excisional biopsy specimens alone and when assessing all specimens together (core and excisional biopsies, $P < .01$). However, when evaluation was restricted to core biopsy specimens only (24 cases evaluated by FISH, 5 with *MYC* gene rearrangement detected), no significant results of association were found. A significant association was not identified between any of the variables and *MYC* gene gain/amplification. Using a cutoff of at least 40% as positive, Table 3 shows the sensitivity and specificity for the various methods of MYC IHC staining evaluation in predicting *MYC* gene rearrangement by FISH analysis. In Table 3, data for core biopsies and excisional specimens are presented together ("all specimens") and separately.

4. Discussion

Original reports in the literature on interpretation of the MYC IHC stain reported high concordance rates among pathologists; however, those studies were based on tissue microarrays and/or review among few pathologists at single institutions [2,11-13]. Subsequent studies have shown less concordance among pathologists when whole tissue sections were scored by a diverse group of pathologists [6,14]. Scoring of the MYC IHC stain can be particularly difficult because of both staining heterogeneity between areas on whole tissue sections and heterogeneity of staining intensity. There is currently no guidance on how to best interpret staining heterogeneity. In our study, we used a systematic approach to retrospective review in an attempt to compensate for staining heterogeneity. In addition, we used digital image analysis as an alternative, and perhaps less biased, method of evaluating MYC IHC staining [15,16]. The percentage of MYC positivity between our manual retrospective review and retrospective review by digital image analysis showed strong correlation (Spearman correlation coefficients of approximately 0.90 for all comparisons). However, when the data were dichotomized to positive or negative for MYC based on a cutoff of 40%, concordance between the 3 methods of evaluation (original IHC interpretation, manual retrospective review, and retrospective review by digital image analysis) was only around 80%, with κ scores showing good agreement. This concordance is similar to that reported among 9 different pathologists in Mahmoud et al [6]. Interestingly, concordance for the core biopsy specimens between the retrospective manual review and retrospective review by digital image analysis in our study was excellent (96%, κ score 0.915), perhaps because of fewer areas evaluated in these specimens or better fixation of the specimen.

In our series, core biopsy specimens were poor predictors of *MYC* gene rearrangement. When we evaluated the core biopsy specimens in isolation, no significant results of association were found between MYC IHC stain and *MYC* gene rearrangement, and sensitivities using a cutoff of 40% positivity were only 0.50 to 0.80 by the various methods used for evaluation. The sensitivity of excisional biopsy specimens was excellent by the original pathologist's interpretation and by manual retrospective review, at the expense of specificity (sensitivities of 1.0 and specificities of 0.63 and 0.33, respectively). The drastic decrease in specificity when evaluated by manual retrospective review (performed in a more systematic fashion and not influenced by morphology and results of additional IHC stains) suggests that, in a clinical setting, our pathologists used other clues to predict an *MYC* gene rearrangement, such as clinical behavior, Ki-67 proliferation index, and aggressive morphology. Alternatively, this could represent the individual bias of the retrospective pathologist reviewer to overestimate percent positivity in this setting. It has been recommended in the literature that MYC IHC staining should not be used as the sole method of *MYC* status evaluation [14]. Our study findings strongly support this conclusion for core biopsy specimens. For excisional biopsy specimens, MYC IHC stain may be a triaging tool if used with great caution. Correlating MYC IHC stain results with morphologic and IHC features suggestive of Burkitt lymphoma or high-grade B-cell lymphoma is invaluable. We found no association between MYC IHC stain and *MYC* gene gain/amplification, although we may not have had enough cases to identify an association.

In our study, digital image analysis had a similar sensitivity for *MYC* rearrangement to the original pathologist's interpretation and the retrospective manual interpretation with a similar specificity to the original pathologist's interpretation when all specimens were evaluated, although it did have a slightly lower sensitivity (0.89) when core biopsy specimens were excluded. The concordance between retrospective review by digital image analysis and the original IHC interpretation was similar to the previously reported concordance among pathologists and the correlation between our manual retrospective review and retrospective review by digital image analysis was strong [6]. These findings indicate that digital image analysis performs comparably to manual analysis and therefore is a viable alternative to manual analysis for interpretation of MYC IHC staining. Our method used the Aperio Nuclear algorithm without manipulation, a strategy that would need confirmation

across other institutions. Advantages to digital image analysis include the possibility of a more standardized approach to interpretation of the IHC stain, the opportunity for a more sophisticated scoring system (eg, taking into account the relative distribution of nuclei with different staining intensities) and the ability of the interpretation to occur at a central location [7]. Disadvantages include the processing power and time required for scanning and analyzing the slides. Our approach to digital image analysis still required manual input to identify viable areas of neoplastic tissue. Applying the nuclear algorithm to the whole slide without manual input would include nonmalignant cells (endothelial cells, nonneoplastic lymphocytes, fibroblasts, etc) in the analysis and introduce artifact from crush, overstaining, tissue folds, and degenerating neoplastic cells.

Beyond its use as a potential triaging tool, expression of MYC protein as determined by IHC staining is a potential prognostic and/or predictive marker. Double expression of MYC and BCL2 proteins in DLBCL, not otherwise specified, is associated with inferior survival in most studies [2,4,12,17-21]. Most studies, but not all, use the cutoff of 40% as indicative of MYC positivity or "overexpression." In our cohort, nearly one-third of our cases had a percent MYC positivity surrounding the cutoff of 40%, and for these cases, our concordance values among the various methods used for evaluation was marginal/poor. This suggests that there is limited utility of this stain as a prognostic or predictive marker using 40% as a cutoff value without further evaluation guidelines. Tsuyama et al [22] performed a systematic review of BCL2 IHC using a scoring system for staining of 0 to 3+, dependent on proportion of cells staining and intensity of staining, and found that a BCL2 3+ score was a significant prognostic factor. A similar scoring system may prove to be valuable for MYC IHC stain interpretation. Digital image analysis may be a robust method to develop a more sophisticated scoring system, standardize the interpretation, and allow for centralized review. Our study was not designed to evaluate clinical outcome; however, we feel that our study demonstrates comparable performance of digital image analysis to manual review, therefore suggesting that future studies can incorporate digital image analysis. Clear guidelines for evaluation of MYC IHC staining interpretation are recommended for future studies regarding its prognostic or predictive value.

To summarize, our results suggest that the MYC IHC stain should not be used as a triaging tool for the *MYC* gene rearrangement in core biopsy specimens. For excisional biopsy specimens, there is potential use as a triaging tool with the caveat that the sensitivity is not 100% (as per the literature and our experience during validation of the MYC IHC stain in our laboratory) and the acknowledgement that there is a low specificity. Results of the MYC IHC stain should always be correlated with morphologic and clinical findings and results of other IHC stains. If the MYC IHC stain is to be used as a prognostic or predictive marker, clear guidelines need to be developed delineating the best method of stain interpretation for this purpose, and digital image analysis should be investigated as a potential method of stain interpretation.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2018.08.019.

References

[1] Gascoyne RD, Campo E, Jaffe ES, et al. Diffuse large B-cell lymphoma, NOS. In: Swerdlow SH, Campo E, Harris NL, et al, editors. World Health Organization classification of Tumours of Haematopoietic and lymphoid tissue. Lyon: IARC; 2017.

[2] Johnson NA, Slack GW, Savage KJ, et al. Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol 2012;30:3452-9.

[3] Petrich AM, Nabhan C, Smith SM. MYC-associated and double-hit lymphomas: a review of pathobiology, prognosis, and therapeutic approaches. Cancer 2014;120:3884-95.

[4] Horn H, Ziepert M, Becher C, et al. MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma. Blood 2013;121:2253-63.

[5] Fitzgibbons PL, Bradley LA, Fatheree LA, et al. Principles of analytic validation of immunohistochemical assays: guideline from the College of American Pathologists Pathology and Laboratory Quality Center. Arch Pathol Lab Med 2014;138:1432-43.

[6] Mahmoud AZ, George TI, Czuchlewski DR, et al. Scoring of MYC protein expression in diffuse large B-cell lymphomas: concordance rate among hematopathologists. Mod Pathol 2015;28:545-51.

[7] Ghaznavi F, Evans A, Madabhushi A, Feldman M. Digital imaging in pathology: whole-slide imaging and beyond. Annu Rev Pathol 2013;8:331-59.

[8] Marcuzzo T, Giudici F, Ober E, Rizzardi C, Bottin C, Zanconati F. Her2 immunohistochemical evaluation by traditional microscopy and by digital analysis, and the consequences for FISH testing. Pathol Res Pract 2016;212:911-8.

[9] Rizzardi AE, Zhang X, Vogel RI, et al. Quantitative comparison and reproducibility of pathologist scoring and digital image analysis of estrogen receptor beta2 immunohistochemistry in prostate cancer. Diagn Pathol 2016;11:63.

[10] Le CT. Applied Categorical Data Analysis and Translational Research. Hoboken, NJ: Wiley; 2010.

[11] Kluk MJ, Chapuy B, Sinha P, et al. Immunohistochemical detection of MYC-driven diffuse large B-cell lymphomas. PLoS One 2012;7:e33813.

[12] Perry AM, Alvarado-Bernal Y, Laurini JA, et al. BCL2 protein expression predicts survival in patients with diffuse large B-cell lymphoma treated with rituximab. Br J Haematol 2014;165:382-91.

[13] Green TM, Nielsen O, de Stricker K, Xu-Monette ZY, Young KH, Moller MB. High levels of nuclear MYC protein predict the presence of MYC rearrangement in diffuse large B-cell lymphoma. Am J Surg Pathol 2012;36:612-9.

[14] Kluk MJ, Ho C, Yu H, et al. Immunohistochemistry to identify MYC-driven B-cell lymphomas in clinical practice. Am J Clin Pathol 2016;145:166-79.

[15] Sander B, de Jong D, Rosenwald A, et al. The reliability of immunohistochemical analysis of the tumor microenvironment in follicular lymphoma: a validation study from the Lunenburg Lymphoma Biomarker Consortium. Haematologica 2014;99:715-25.

[16] Aeffner F, Wilson K, Martin NT, et al. The gold standard paradox in digital image analysis: manual versus automated scoring as ground truth. Arch Pathol Lab Med 2017;141:1267-75.

[17] Rosenthal A, Younes A. High grade B-cell lymphoma with rearrangements of MYC and BCL2 and/or BCL6: double hit and triple hit lymphomas and double expressing lymphoma. Blood Rev 2017;31:37-42.

[18] Hu S, Xu-Monette ZY, Tzankov A, et al. MYC/BCL2 protein coexpression contributes to the inferior survival of activated B-cell subtype of diffuse large B-cell lymphoma and demonstrates high-risk gene expression signatures: a report from the International DLBCL Rituximab-CHOP Consortium Program. Blood 2013;121:4021-31 [quiz 4250].

[19] Green TM, Young KH, Visco C, et al. Immunohistochemical double-hit score is a strong predictor of outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol 2012;30:3460-7.

[20] Valera A, Lopez-Guillermo A, Cardesa-Salzmann T, et al. MYC protein expression and genetic alterations have prognostic impact in patients with diffuse large B-cell lymphoma treated with immunochemotherapy. Haematologica 2013;98:1554-62.

[21] Scott DW, Mottok A, Ennishi D, et al. Prognostic significance of diffuse large B-cell lymphoma cell of origin determined by digital gene expression in formalin-fixed paraffin-embedded tissue biopsies. J Clin Oncol 2015;33:2848-56.

[22] Tsuyama N, Sakata S, Baba S, et al. BCL2 expression in DLBCL: reappraisal of immunohistochemistry with new criteria for therapeutic biomarker evaluation. Blood 2017;130:489-500.