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SHORT REPORT

Does the proliferation fraction help identify mature B cell lymphomas with double- and triple-hit translocations?

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Aims: The entity 'B cell lymphoma, unclassifiable, with features intermediate between diffuse large B cell lymphoma (DLBCL) and Burkitt lymphoma (BL)' refers to B cell neoplasms that share overlapping characteristics of BL and DLBCL. A subset of these 'grey-zone lymphomas' possesses *C-MYC* and *IGH* translocations but, in addition, contains additional rearrangements of *BCL2* and/or *BCL6* genes. The aim of this study was to investigate if the proliferation fraction by Ki67 immunostaining can be used to identify such double-/triple-hit lymphomas.

Methods and results: We studied 492 cases of mature aggressive B cell neoplasms by histology, immunohistochemistry and interphase fluorescence *in-situ* hybridization (FISH) using break-apart probes against *C-MYC*, *BCL2*, *BCL6*, *IGH*, *MALT1*, *PAX5* and *CCND1*. Forty Burkitt lymphomas and 28 cases of MYC^+ double-/triple-hit lymphomas were identified. Of the latter, 77% and 54% displayed proliferation fractions exceeding 75% and 90%, respectively. With a cut-off of >75% by Ki67 immunostaining, the sensitivity and specificity for detection of MYC^+ double/triple translocations was 0.77 and 0.36. Raising the proliferation fraction criterion to >90% improved the specificity to 0.62 at the expense of a low sensitivity of 0.54.

Conclusions: Immunostaining for Ki67 is not a useful approach to prescreen B cell lymphomas for MYC^+ double/triple translocations.

Keywords: chromosomal translocation, diffuse, Ki-67 antigen, large B cell, lymphoma

Abbreviations: BL, Burkitt lymphoma; DLBCL, diffuse large B cell lymphoma; FISH, fluorescence in-situ hybridization

Introduction

The accurate classification of aggressive mature B cell neoplasms is important, because the prognosis and chemotherapeutic regimens used are quite different. Burkitt lymphoma has an aggressive clinical behaviour, but responds well to intensive chemotherapy such as HyperCVAD or CODOX-M/IVAC. In contrast, diffuse large B cell lymphoma (DLBCL) has a poorer overall survival compared to Burkitt lymphoma, and is usually treated with R-CHOP chemotherapy.

Demonstration of the characteristic t(8;14), t(2;8) and t(8;22) translocations involving *C-MYC* and an immunoglobulin gene is characteristic of BL. As a surrogate marker, a high proliferation fraction of 'almost 100%' by Ki67 immunostaining had been suggested,[1] but a subset of DLBCL with morphological

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and phenotypic overlap with BL also possesses a high proliferation fraction.

Currently, demonstration of the *C-MYC/IGH* translocation is almost mandatory in the diagnosis of BL, usually by karyotyping or interphase fluorescence *in-situ* hybridization (FISH). However, *C-MYC* translocation is not limited to BL. It is known that DLBCL can also possess *C-MYC* translocations, but often in such instances the translocation partner may be a non-immunoglobulin gene[2] or there is an additional second or third translocation, usually involving *BCL2* or *BCL6.*[3–6] Such cases of MYC^+ double- or triple-hit lymphomas usually show higher proliferation fractions[7] and a poorer prognosis when treated with either BL- or DLBCL-type therapies.

In contrast, true BL cases possess *C-MYC* translocations to an immunoglobulin gene only, without additional alterations in *BCL2* and *BCL6* as seen in MYC^+ DLBCL. This is also confirmed by comparative genomic hybridization data, which have demonstrated that BL is genetically stable while DLBCL with *C-MYC* translocations tend to have more alterations at the genomic level.[4,8]

It may now become necessary for the pathologist to interrogate for translocations of not only *C-MYC* and *IGH*, but also *BCL2* and *BCL6* when confronted with the differential diagnosis of BL versus DLBCL. In addition, if no *IGH* rearrangement is noted in a lymphoma with *C-MYC* translocation, rearrangement of *IGK* and *IGL* genes has to be ascertained. Even when the diagnosis is clearly that of DLBCL, there may be a need to separate out cases of MYC^+ double-/triple-hit lymphomas that do not respond well to standard R-CHOP therapy. This poses a significant burden on the resources of the pathology laboratory, and the question has been raised as to whether Ki67 immunostaining can help to predict the presence of MYC^+ double-/triple-hit lymphomas. Would it be feasible to prescreen DLBCL for cases with high proliferation fraction for further analysis by interphase FISH to diagnose double-/triple-hit lymphomas?

Methods

A total of 492 cases of DLBCL, grey-zone lymphomas and BL from 2004 to 2011 in the archives of the Department of Pathology, Singapore General Hospital were characterized by review of histology, an extended panel of immunostains (including those for BCL2, BCL6, CD10, MUM1, FoxP1, LMO2 and Ki67) and classified according to Hans' criteria.[9] Where phenotyping by Hans' criteria remained indeterminate (e.g. $CD10^-$ $BCL6^-$ $MUM1^-$), expression of germinal centre marker LMO2 versus post-germinal centre marker FoxP1 was used to make the final arbitration. Assessment of Ki67 immunostaining was performed by a single observer and divided into four categories (<50%, >50–75%, >75–90%, >90%), based on the percentage of cells stained. To assess the degree of inter- and intraobserver error, Ki67 staining in 121 cases was graded independently by two pathologists (K.E. and T.S.Y.). To assess the degree of intraobserver agreement, the same cases were reassessed by the study pathologist (K.E.) more than 3 months apart, blinded to results of the previous assessment. Statistical analysis for inter- and intraobserver agreement was performed and the weighted kappa statistics calculated using SPSS software (IBM, Armonk, NY, USA). All cases were also investigated by interphase FISH using break-apart probes to *BCL2*, *BCL6*, *IGH*, *C-MYC*, *CCND1*, *MALT1* and *PAX5*. In the event of a *C-MYC* translocation but intact *IGH* gene, FISH for *IGK* and *IGL* was performed.

Results

Forty cases of Burkitt lymphoma were identified, of which sufficient material was available in 28 cases for both immunohistochemistry and complete FISH analysis. All cases tested displayed a high proliferation fraction of >95% and possessed *C-MYC* translocation to an immunoglobulin gene.

Of all cases of DLBCL, 46 showed double or triple translocations in addition to *IGH* (Table 1; Figure 1). Of these, 28 showed *C-MYC* translocations (14 cases involving *C-MYC* and *BCL2;* nine involving *C-MYC* and *BCL6*; and five triple-hit cases). The remaining 18 double-hit lymphomas lack *C-MYC* translocations and were not considered further in this study, which focussed only on MYC^+ double-/triple-hit lymphomas.

MYC^+ double-/triple-hit lymphomas showed male predominance, comprising 19 males and nine females (M:F = 2:1) with a median age of 57.8 years (range 31–84). Nodal disease was seen in 43% and, of the majority that presented in extranodal sites, the head and neck region (14%) and gastrointestinal tract (11%) were most commonly involved.

Assessment of a starry-sky architecture and prominence of nucleoli in neoplastic cells did not distinguish double-/triple-hit lymphomas from DLBCL without these alterations. In terms of phenotype, using Hans' criteria there were slightly more MYC^+ double-/triple-hit lymphomas in the germinal centre B cell-like (GCB) subgroup (61%) than in the activated B cell-like (ABC) subgroup (39%). However, with additional markers LMO2 and FoxP1, they were divided more evenly between GCB (52%) and ABC (48%) phenotypes.

Table 1. Summary of genetic alterations and proliferation fraction by Ki67 immunostaining

Lymphoma	Alteration	Type	Subtotal	Total
MYC$^+$ double- and triple-hit lymphomas	Double-hit	*MYC/BCL2*	14	28
		MYC/BCL6	9	
	Triple-hit	*MYC/BCL2/BCL6*	4	
		MYC/BCL2/PAX5	1	
MYC$^-$ double- and triple-hit lymphomas	Double-hit	*BCL2/BCL6*	14	18
		PAX5/BCL2	1	
		PAX5/BCL6	3	
Burkitt lymphoma				40
DLBCL without double- or triple-hit translocations				406
Total				492
Proliferation fraction using Ki67 > 75% as cut-off				
Lymphoma		Ki67 < 75%	Ki67 > 75%	Total
DLBCL with *MYC*$^+$ double-/triple-hit translocations		6	20	26
DLBCL without *MYC*$^+$ double-/triple-hit translocations		148	258	406
Total		154	278	432
Proliferation fraction using Ki67 > 90% as cut-off				
Lymphoma		Ki67 < 90%	Ki67 > 90%	Total
DLBCL with *MYC*$^+$ double-/triple-hit translocations		12	14	26
DLBCL without *MYC*$^+$ double-/triple-hit translocations		251	155	406
Total		263	169	432

DLBCL: diffuse large B cell lymphoma.

DLBCLs with *BCL2* and *C-MYC* translocations tended to show the GCB phenotype (71%), while cases with *BCL6* and *C-MYC* translocations tended to show the ABC phenotype (89%).

The proliferation fraction in *MYC*$^+$ double-/triple-hit lymphomas ranged from 30 to 100% and was generally high, exceeding 75% in 20 of 26 cases (77%). In fact, 14 of 26 cases (54%) displayed a very high proliferation fraction of >90%. Conversely, proliferation fractions of >75% and >90% were seen in 64% and 38% of DLBCL without double/triple translocations, respectively (range 20–95%). The lowest proliferation fraction of 30% was seen in three (12%) of 26 *MYC*$^+$ double-/triple-hit lymphomas, while cases without *MYC*$^+$ double-/triple-hit translocations displayed the lowest proliferation fraction of 20% focally. In other words, it is not possible to define a proliferation fraction below which all cases of double-/triple-hit translocations are excluded. Interobserver agreement was 90.5% with a weighted kappa value of 0.677, indicating good agreement. The percentage of intraobserver agreement was 83.26%, with a weighted kappa statistic of 0.386.

Using a proliferation fraction cut-off of >75% to predict *MYC*$^+$ double-/triple-hit lymphomas, a sensitivity of 0.77 was achieved, with a low specificity of 0.36. Raising the proliferation fraction cut-off to >90% had the effect of increasing the specificity to 0.62, but dropping the sensitivity to 0.54.

Conclusion

Diffuse large B cell lymphomas with *MYC*$^+$ double-/triple-hit translocations present commonly in older

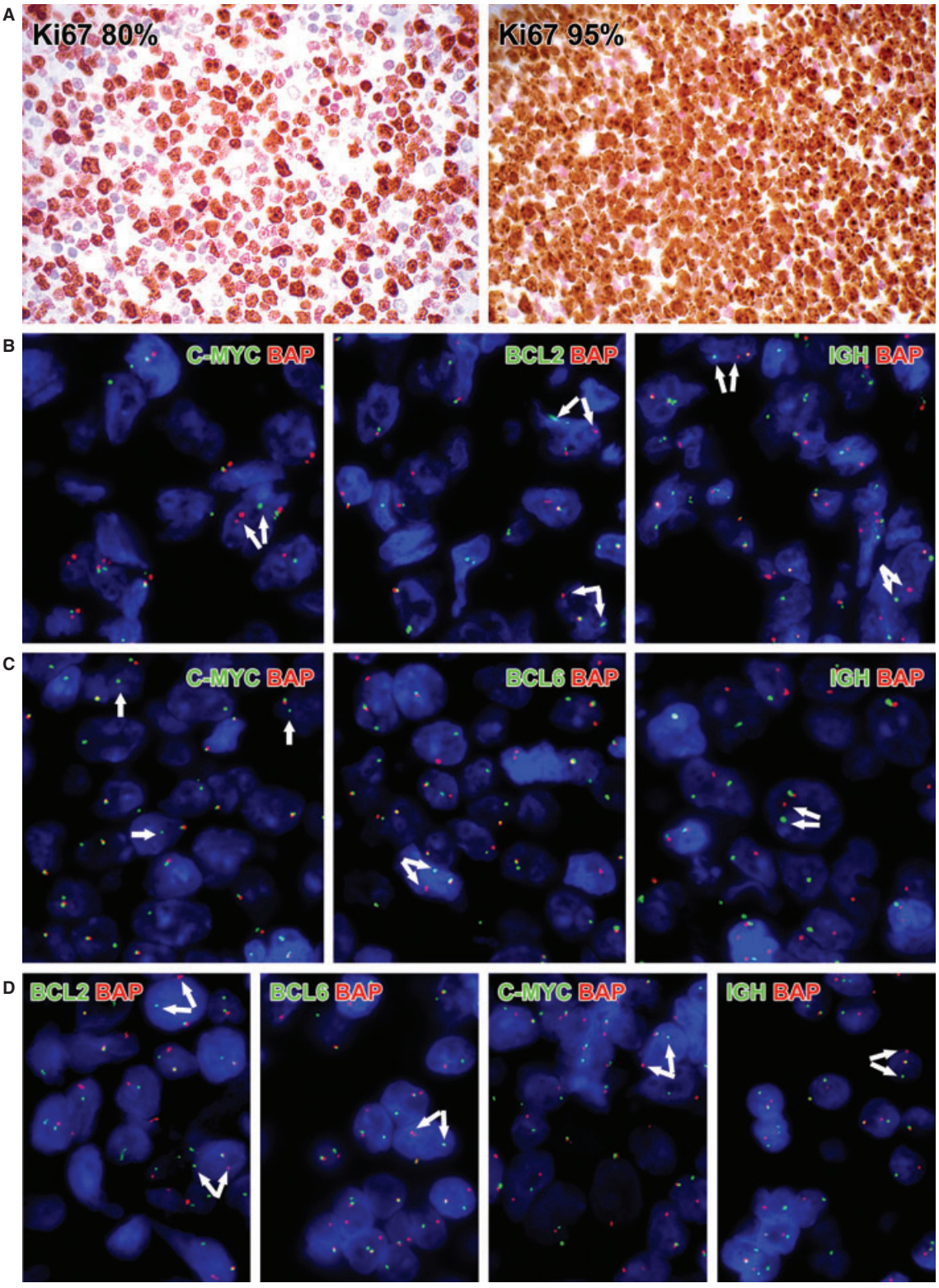

Figure 1. **A**, Diffuse large B cell lymphoma (DLBCL) with proliferation fractions of 80% and 95% by immunostaining for Ki67. **B**, Interphase fluorescence *in-situ* hybridization (FISH) using break-apart probes in DLBCL with double-hit translocations involving *C-MYC*, *BCL2* and *IGH*. **C**, DLBCL with double-hit translocations showing rearrangements of *C-MYC*, *BCL6* and *IGH*. **D**. FISH using break-apart probes in this case shows translocations of *BCL2*, *BCL6*, *C-MYC* and *IGH*, in keeping with a triple-hit lymphoma.

males. Nodal disease was seen in 43% and the most common extranodal sites were the head and neck region and gastrointestinal tract. Neither immunophenotype by Hans' criteria, a starry-sky appearance nor the presence of prominent nucleoli predicted DLBCL with multiple translocations. The most common alterations in MYC^+ double-hit lymphomas contained *C-MYC*, *BCL2* and *IGH* translocations, followed by *C-MYC*, *BCL6*, *IGH* translocations, while the most common triple-hit translocation involved *BCL2*, *BCL6*, *C-MYC* and *IGH* genes.

Assessment of the proliferation fraction by Ki67 immunostaining may be hampered by differing fixation conditions, large numbers of tumour-associated inflammatory cells, prolonged storage and storage conditions. It has also been shown that Ki67 staining suffers from high interlaboratory variability,[10] although the interobserver agreement achieved in this study was good. However, to be useful as an assay to prescreen DLBCLs for detection of MYC^+ double-/triple-hit translocations, the test must be sufficiently sensitive to identify most cases for confirmation by FISH. Using a cut-off criterion of >75% by Ki67 immunostaining, the sensitivity of 0.77 is fair, although as many as a quarter of cases will be missed. However, the specificity of only 0.36 means that a large number of translocation-negative cases will be interrogated unnecessarily by FISH. This will not result in very significant savings. Conversely, raising the bar to >90% by Ki67 immunostaining improves the specificity somewhat to 0.62 at the cost of a reduced sensitivity of 0.54. Given the poor sensitivity and specificity data, we conclude that Ki67 immunostaining is not suitable as a means to prescreen DLBCL for MYC^+ double-/triple-hit lymphomas.

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