Anti-myelocytomatosis tag antibody detects myelocytomatosis oncogene expression in Burkitt lymphoma

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Background: The immunohistochemical (IHC) detection of *myelocytomatosis oncogene* (*MYC*) is a crucial step in the diagnosis and prognosis of Burkitt lymphoma (BL). Sections of the MYC protein are routinely used as tags in protein precipitation experiments to assist with the isolation of proteins without antibodies. However, it is unknown if the tag antibodies can also be used for BL diagnosis.

Aim: This project aimed to determine whether the MYC tag 9E10 antibody can be used to detect MYC overexpression because of *MYC* translocation in BL cases.

Setting: Charlotte Maxeke Johannesburg Academic Hospital, South Africa.

Methods: Immunohistochemical staining for 9E10 was optimised and used to stain 10 BL with known *MYC* translocation status to calculate sensitivity, specificity and predictive values.

Results: Staining of the BL cases generally produced a 'very weak' (70%) and weak-moderate (18.2%) staining patterns with a staining extent of 1+ (36%) and 3+ (27%). Of the 10 samples, 6 (60%) showed a positive MYC protein expression by IHC. In comparison, 7 (70%) samples indicated *MYC* gene rearrangements. There were 5 (50%) cases with both MYC IHC expression and gene translocations and 2 (20%) cases that were negative for both MYC IHC and gene rearrangements.

Conclusion: The authors demonstrate that the 9E10 MYC tagged antibody may be used to detect *MYC* gene expression with a sensitivity of 71% and a specificity of 67%. In addition, the positive predictive value (PPV) and negative predictive value (NPV) varied according to IHC staining cut-offs. Immunohistochemical expression does not perfectly correlate with translocation status because of inconsistencies with IHC interpretation.

Contribution: *MYC* gene rearrangements are present in nearly all BL cases. Finding more affordable and convenient ways to predict the presence of *MYC* gene rearrangements is of utmost importance, given the lack of financial resources in our continent. This study shows that the 9E10 antibody, commonly used in protein tagging experiments, may also be used to predict *MYC* gene rearrangements in BL.

Keywords: immunohistochemistry; fluorescence in situ hybridisation; 9E10; optimisation; *MYC* rearrangement; antibodies; Burkitt lymphoma.

Introduction

The *myelocytomatosis oncogene* (*MYC*) gene translocation is one of the hallmark diagnostic markers of Burkitt lymphoma (BL).[1] Burkitt lymphoma mainly arises from the reciprocal translocation of the *MYC* gene with the heavy chain locus from chromosome 8 to 14, thus leading to its constitutive expression.[2] The presence of *MYC* translocation is detected using fluorescence in situ hybridisation (FISH) technology, which is not widely available in resource-limited settings.[3] As the translocation leads to overexpression of MYC protein, numerous studies have sought to associate the expression of MYC protein with the presence or absence of *MYC* gene translocation using the readily available immunohistochemistry.[3,4,5] However, MYC protein expression does not always correlate with *MYC* translocation because of discordance caused by inter-observer variability and a lack of standardisation in immunohistochemical (IHC) scoring.[6]

The MYC protein belongs to a family of transcription factors characterised by the presence of the helix-loop-helix and leucine zipper structural motifs. The MYC family of proteins includes the

related C-MYC, L-MYC and N-MYC.[7] The helix-loop-helix motif allows it to bind to DNA, while the leucine zipper motif allows it to bind to other translocation factors.[8]

The most common hetero-dimerisation partner for MYC is MAX, which binds to E-box regulatory DNA elements to affect transcriptional control of genes and ultimately induce cell proliferation and inhibition of cellular differentiation.[7]

Immunohistochemistry represents a way to build the distribution and localisation of molecular markers within the proper tissue context. It is a powerful tool that provides important diagnostic, prognostic and predictive information supplemental to the morphological assessment of the tissues.[9]

Antibodies are molecules that bind to specific epitopes or regions within proteins. Linear epitope sequences average between six and nine amino acids.[10] Epitope tagging is fusing an epitope to recombinant proteins by genetic engineering. This technique offers the ability to detect, purify and characterise newly discovered proteins for which specific antibodies are not available. The most commonly used protein tags include the MYC, FLAG and His tags.[11] The MYC tag sequence, EQKLISEEDL, is derived from the loosely defined epitope of the monoclonal antibody 9E10.[12]

Several studies have previously detected MYC protein expression in B-cell lymphomas using the rabbit monoclonal Y69 antibody.[3,4,5] Our study aimed to determine whether the 9E10 commercial antibody can be used to detect MYC protein expression in BL samples.

Methods

Sample collection

A SNOMED search on the NHLS database was conducted through the TrakCare® (Cambridge, United Kingdom) Lab web viewer using snomed codes (M-96873, M-80003, M-95903), to obtain records of BL cases from the Charlotte Maxeke Johannesburg Academic Hospital from 2014 to 2020. The diagnosis of the cases was not reviewed according to the 2016 WHO guidelines.[13] The diagnostic criteria were based on morphological and immunophenotypic expression. Burkitt Lymphoma-like cases, those without FISH results and missing tissue blocks were excluded.

Optimisation of MYC antibodies

A BL (containing *MYC* translocation by FISH) tissue block was sectioned at 5 μm and stained with the 9E10 mouse monoclonal antibody (Invitrogen, ThermoFisher Scientific, United States). The manufacturer provided proof of validation on the technical specifications insert. Various antigen retrieval buffers (pH 9 and pH 6), antibody dilutions (1:50 and 1:100) and linker conditions (present or absent) were tested for the antibody. Visualisation was

achieved with the Dako Envision FLEX antibody (Agilent, United States). All IHC staining was performed on the Dako Autostainer Link 48 instrument (Agilent, United States).

Scoring systems

The optimised IHC staining conditions of the most sensitive antibody were used to stain sections from the 10 BL cases. A single pathologist assessed the staining pattern of the BL tissue section. The MYC IHC staining was scored using the Geisinger method,[14] which uses a scoring system based on the extent and intensity of the stain.

The extent of the stain was recorded as:

0 (< 5% of the target cells stained), 1+ (5% – 25% of the target cells stained), 2+ (26% – 50% of the target cells stained), 3+ (51% – 75% of the target cells stained), or 4+ (> 75% of the target cells stained).

The staining signal was recorded as weak, intermediate or strong. A strong signal can be easily seen on low magnification; a weak signal is usually observed on high magnification; an intermediate signal borders between a strong and a weak staining signal.

Data analysis

To determine the association of MYC IHC with rearrangement status, sensitivity, specificity positive predictive value (PPV) and negative predictive value (NPV) were calculated according to established methods.[15] Positive cases, based on staining extent, were stratified, to determine the effect of IHC cut-off values on sensitivity, specificity and predictive values:

- Sensitivity = true pos/(true pos + false neg) × 100
- Specificity = true neg/(true neg + false pos) × 100
- PPV = true pos/(true pos + false pos) × 100
- NPPV = true neg/(true neg + false neg) × 100

- 'True positive' were defined as those with positive FISH status and positive IHC status
- 'True negative' were defined as those with negative FISH status and negative IHC status
- 'False positive' were defined as those with negative FISH status and positive IHC expression
- 'False negative' were defined as those with positive FISH status and negative IHC expression

Ethical considerations

This study was conducted according to the Declaration of Helsinki guidelines. Ethics approval was obtained from the University of the Witwatersrand Human Research Ethics Committee (R14/49). The need for written informed consent from patients was waived.

TABLE 1: Summary of the demographic and clinical characteristics of the study cohort.

Feature	Number of cases	%
Gender		
Female	6	60.00
Male	4	40.00
Nodality		
Extra nodal	8	80.00
Nodal	2	20.00
Anatomical site		
Female genital tract	1	12.50
Gastrointestinal tract	1	12.50
Head and neck	2	25.00
Left nasal mass	1	10.00
Nasal mass	1	12.50
Pancreas	1	12.50
Skin and soft tissue	1	12.50
Lymph node	2	20.00
HIV status		
RVD −	3	30.00
RVD +	7	70.00
***MYC* translocation status**		
MYC +	7	70.00
MYC −	3	30.00

Note: Mean age ± s.d. = 23.7 ± 13.90; range (years) 2–42.

s.d., standard deviation; RVD retroviral disease; MYC, myelocytomatosis oncogene.

TABLE 2: The IHC optimisation staining results of the 9E10 anti-MYC antibody.

IHC condition	1:50	1:100
MYC 9E10 mouse monoclonal antibody		
Low pH (6) with no linker	Negative	Negative
Low pH (6) with linker	Weakly positive	Weakly positive
High pH (9) with no linker	Weakly positive	Negative
High pH (9) with linker	Weakly positive	Positive

IHC, immunohistochemical; MYC; myelocytomatosis oncogene.

Results

Ten BL cases conformed to the inclusion criteria of this study from the TrakCare® database search conducted during the study period using both pathological and FISH data (Table 1). The mean age of the patients was 23.7 ± 13.9 years with 60% females and 40% males. Most patients (80%) presented at extra-nodal sites with the head and neck region being the most prevalent at 25%. The HIV status was positive in 70% of the patients. There was negative staining at 1:50 and 1:100 antibody concentrations with the low pH buffer with no linker. Adding a linker produced weak staining with low pH buffer at both antibody concentrations (Table 2). The high pH buffer produced negative staining with the 1:100 antibody concentration without a linker. Increasing the antibody concentration without a linker to 1:50 produced a weakly positive staining pattern. Adding a linker produced a positive staining pattern with the 1:100 antibody concentration (Table 2, Figure 1).

The optimised conditions of the 9E10 MYC antibody were then used to stain sections from the 10 BL cases retrieved from archives. The staining signal of the 9E10 antibody ranged from very weak to moderately strong (Table 3). The majority of the cases produced a 'very weak' staining pattern (70%, *n* = 7), those producing a 'weak-moderate'

staining pattern were 20% (*n* = 2) and those with weak and moderately strong staining patterns were 10% (*n* = 1), respectively.

The extent of the stain ranged from 0 to 4+ (Table 3). The majority of the cases stained at 1+ (40%, *n* = 4), 3+ (30%, *n* = 3) and 2+ (20%, *n* = 2).

The effect of the IHC cut-off value on the sensitivity, specificity and predictive values were analysed (Table 4). It was observed that sensitivity decreased as the IHC cut-off values were increased. The sensitivity decreased from 86% to 43% when the IHC cut-off values were increased from 1+ to 3+. The specificity value increased from 0% to 67% when the cut-off values increased from 1+ to 2+. The PPV was 67% at 1+ but increased to 83% at 2+ cut-off then decreased to 75% at 3+. The NPV was 0% at 1+, increased to 50% at 2+ then decreased to 33.3% at 3+.

Discussion

The overexpression of *MYC* is one of the diagnostic hallmarks in the diagnosis of BL.[1] Many studies have sought to determine whether IHC may be a surrogate for FISH analysis in *MYC* detection.[3,4,6] Several monoclonal antibodies were previously isolated against MYC, which recognise the protein's N- and C-terminal regions.[16] The most widely used has been the mouse monoclonal antibody 9E10 with the target epitope is now known to be the C-terminal 10-amino acid sequence EQKLISEEDL.[12] The antibody has been used in immunofluorescence,[17] immunoprecipitation and immunoblotting studies.[12] Previous IHC use in prostate cancer and melanomas have yielded excellent results that are largely consistent with the location of MYC.[18,19] Recently, antibodies targeting the MYC N-terminus have been produced: N-262 (rabbit polyclonal) and Y69 (rabbit monoclonal). Both gave excellent results largely consistent with molecular studies regarding the localisation of MYC in normal and tumour tissue.[20,21] The apparent discrepancy between staining patterns determined by using the 9E10 antibody with those determined by the use of Y69 antibody or N-262 antibody has led to uncertainty regarding the localisation of MYC in normal and tumour tissue. This study showed that the staining pattern produced by the 9E10 antibody was confined to the nucleus of the BL tumour (Figure 1). In addition, the BL cases used were tested for *MYC* gene translocation to confirm the overexpression of MYC protein.

The antibody concentration of the 9E10 used in this study was comparable with those of the Y69 antibody.[4,22] The disadvantage of the 9E10 antibody is that it generally produced a very weak staining pattern with up to 75% tumour cells staining (Table 3). The antibody's sensitivity, specificity and predictive values for the antibody varied depending on the IHC positivity cut-off value (Table 4). This phenomenon was previously observed in the study by Lynnhtun.[1] Generally, it seems higher IHC cut-off values correlate with *MYC* translocation in B-cell
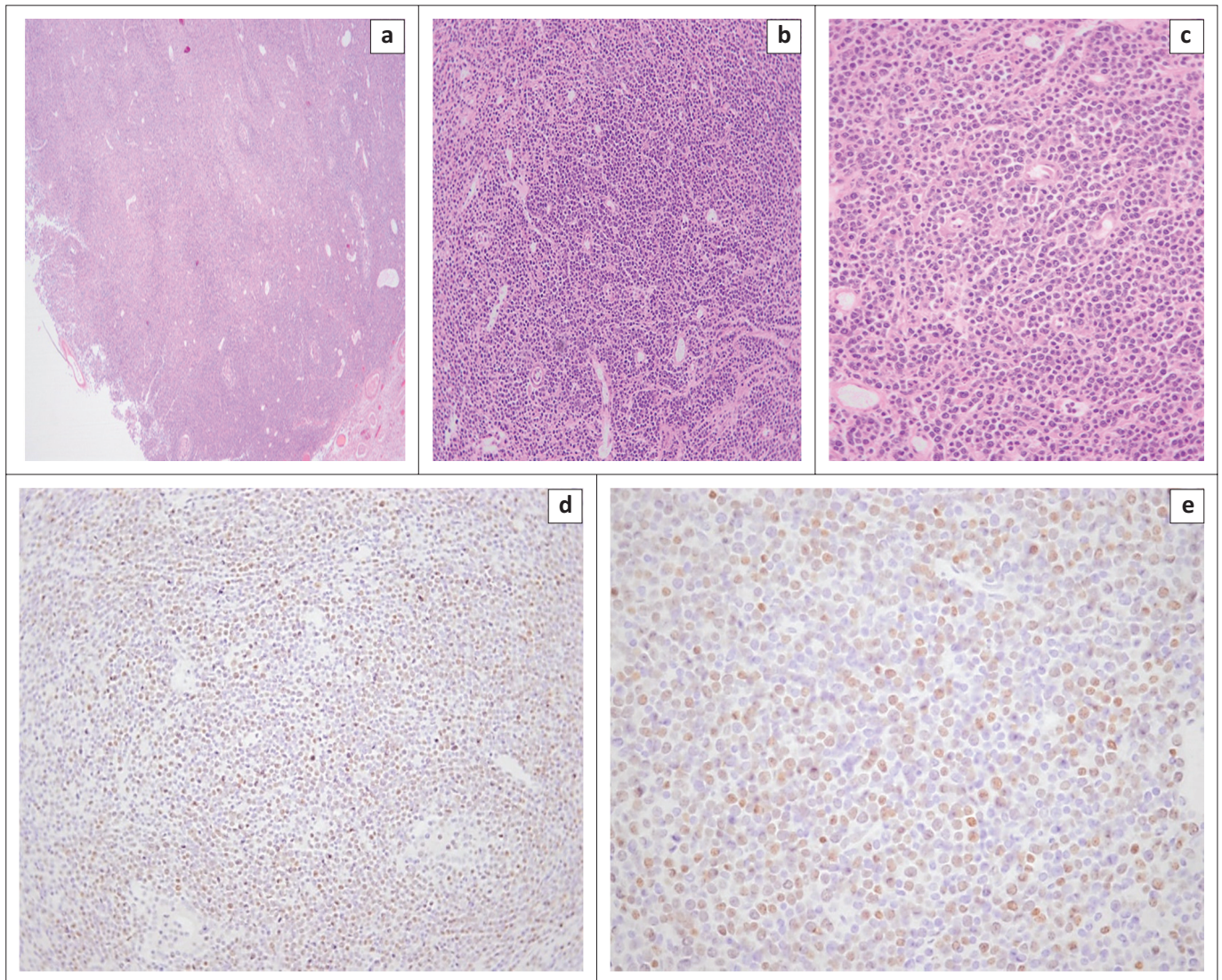
FIGURE 1: Hematoxylin and eosin (H&E) stain of positive high-grade non-Hodgkin lymphoma cell used as a control with positive FISH results for *MYC* rearrangement. (a) 4× magnification, (b) 10× magnification and (c) 40× magnification all stained by the routine H&E stain. (d, e) Showing positive results for the MYC mouse monoclonal antibody 9E10 immunohistochemical stain at 10× and 40× magnification. There is crisp nuclear staining of the cells.

TABLE 3: A summary of the staining intensity and extent of the 10 Burkitt lymphoma cases.

Staining feature	Count	%
Staining intensity		
Very weak	6	60
Weak	1	10
Weak-moderate	2	20
Mod-strong	1	10
Staining extent		
0	1	10
1+	3	30
2+	2	20
3+	3	30
4+	1	10

TABLE 4: Changes in sensitivity, specificity and predictive values depending on the immunohistochemical positivity threshold.

Test characteristic	Immunohistochemical positivity cut-off values		
	1+ (%)	2+ (%)	3+ (%)
Sensitivity	86	71	43.00
Specificity	0	67	67.00
PPV	67	83	75.00
NPV	0	50	33.30

PPV, positive predictive value; NPV, negative predictive value.

lymphoma cases, thus suggesting a higher proportion of staining is required for correlation with *MYC* translocation.[1,4,5,6] However, MYC protein may be overexpressed in *MYC* translocation negative cases.[4] This suggests that other pathways may influence the overexpression of *MYC* in B-cell lymphomas.[5] A study by Valera et al. demonstrated multiple genetic pathways that culminate in MYC overexpression in B-cell lymphomas, including the loss and gain of copies.[23]

The IHC staining performance of antibodies may be affected by factors such as type of fixation, fixation time, time in storage and the storage conditions, which may be true for 9E10.

Limitations

This study was limited by the small sample size, which may have limited the observed staining pattern results. In addition

to this, the IHC stains were analysed by a single pathologist, which may have introduced interpretation bias.

Conclusion

This study was the first to correlate MYC immunohitsochemical staining with *MYC* translocation status using the 9E10 monoclonal antibody in BL cases. It was found that the antibody showed nuclear staining in tumour cells, although with a weak staining pattern. It was further demonstrated that the antibody correlates with *MYC* translocation with a sensitivity of 71% and a specificity of 67%. In addition, the PPV and NPV were 83% and 50%, respectively.

The results of this study support the fact that IHC expression does not perfectly correlate with FISH translocation status because of variations in protein expression interpretation. A standardised approach to IHC interpretation needs to be implemented in order to better utilise this technique clinically.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

N.B.S. was involved in project conceptualisation, data extraction, data analysis and wrote the original draft of the manuscript. N.N. was involved in project conceptualisation, data extraction, data analysis, supervision and wrote the original draft of the manuscript. P.S.M. was involved in project conceptualisation, data extraction, data analysis, supervision and reviewed and edited the manuscript.

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Disclaimer

The authors declare that the views expressed in the submitted article are their own and not an official position of the institution or funder.

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