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B-Cell Non-Hodgkin's Lymphoma characterisation by immunohistochemistry in Indigenous black Zambians

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Abstract

B-cell Non-Hodgkin's lymphomas are a heterogeneous disease entity, accounting for about 21 different subtypes and 85% of all NHLs. This heterogeneity often makes clinical management of these patients difficult. Currently very little is known about the immunophenotype of the B-cell NHLs in Zambia. Immunohistochemistry plays a crucial role in the proper classification, determination of prognosis and an optimum therapeutic schedule in these lymphoid malignancies. This study was aimed at characterizing the immunohistochemical expression of 5 proteins (CD10, BCL-2, BCL-6, CD23 and Cyclin D1) in 28 cases of B-cell NHLs. This was a cross-sectional study conducted to evaluate 28 conveniently sampled B-cell NHLs formalin fixed paraffin embedded tissues from the year 2012 at the University Teaching Hospital for a six month period. The following panels of primary antibodies were used: CD10, CD23, CyclinD1, BCL-2 and BCL-6 (Dako, Glostrup, Denmark). The Labelled Streptavidin Binding (LSAB) staining was used to amplify and view the reaction. Fisher's exact test was used to indicate statistical significance of the findings. The B-cell NHLs showed positive expression to the markers as follows; Diffuse large B cell lymphoma CD10 3/11(27.4%), BCL-2 6/11(54%) and BCL-6 1/11(9.1%). Burkitt lymphoma, CD 10 4/5(80%) and BCL-6 4/5 (80%). Small lymphocytic lymphoma, CD10 1/2(50%), BCL-2 1/2(50%) and, CD 23 2/2(100%). Mantle cell lymphoma CD10 ¼(25%), BCL-2 3/4(75%) and Cyclin D1 4/4(100%). Follicular lymphoma was CD10 1/2(50%). About 4 cases (14.3%) of the B-cell NHL were unclassified and they were positive only for CD10 3/4(75%). The B-cell NHLs has shown variable expression of the surface membrane proteins. Specifically the over expression of BCL-2 and reduced expression of CD10 and BCL-6 by DLBCLwhich accounted for the majority of the B-cell NHLs is suggestive of a very aggressive NHL prevailing among indigenous black Zambians.

Key words: B-cell Non-Hodgkin's lymphoma, Immunohistochemistry

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Introduction

B-cell Non-Hodgkin's lymphomas are a heterogeneous disease entity, accounting for about 21 different subtypes and 85% of all NHLs [1, 2]. However, each of these entities is also heterogeneous with regards to clinical presentation and outcome, often making clinical management of these patients difficult [3].

Immunohistochemistry

CD markers

Advances in immunology have led to the classification of several cellular subtypes including T-cells, B-cells, and antigen-presenting cells that play a role in the immune response. With many cells having similar morphological characteristics, the distinction of subtypes has advanced with the identification of cell lineages using monoclonal

antibodies targeted at surface proteins. Immunophenotyping has become a powerful tool in classifying and organizing several types of diseases, from infectious to inflammatory to neoplastic [4]. The abbreviation CD which stands for human cluster of differentiation markers, or cluster of designation (CD) markers, are a classification system for monoclonal antibodies against cell surface molecules on leukocytes and antigens from other cells. Currently, more than 400 CD markers have been identified, although not all of them are of diagnostic value [5].

CD 10

The CD10 antigen is a cell-surface metallopeptidase that splits peptide bonds on the amino side of hydrophobic amino acids. It was initially called as the common acute lymphoblastic leukaemia antigen (CALLA). The antigen CD10 is normally expressed by precursor B-lymphocytes and by lymph node germinal centers, reflecting a biphasic pattern of CD10 expression in B-cell differentiation [6]. Physiologically CD10 has been shown to down regulate a number of certain peptides such as bombesin, endothelin-1, and basic fibroblast growth factor by reducing the amount of peptide available for receptor binding and signal transduction and also by forming protein complexes with other proteins such as Lyn kinase. This has been shown to provide tumour suppressive activities [7]. Among lymphomas, CD10 is primarily seen in Follicular lymphoma, Burkitt lymphoma, lymphoblastic lymphoma/leukaemia, and a subset of diffuse large B-cell lymphomas [8].

CD23

The antigen CD23 is human leukocyte differentiation protein and a key molecule for the activation and growth of B-cells. It is the low-affinity receptor for IgE, hence also called FCER2. The condensed molecule can be secreted, and then functions as a potent mitogenic growth factor and is located on human chromosome 19 [9]. The expression of CD23 is seen in a variety of cell types including activated B-cells and a subset of follicular dendritic cells; those in the light zone of the follicle center [10]. CD23 expression is present in the majority of CLL/SLL cases, and its absence in MCL has diagnostic utility as these are both CD5 positive [11]. In CLL/SLL, the level of expression may be variable and CD23 expression appears to be greater in the larger cells seen in proliferation centers. The lack of staining for follicular dendritic meshworks using CD21 or CD23 may be helpful in identifying diffuse areas in follicular lymphoma [10].

BCL-2

The BCL-2 family of proteins comprises both antagonists and agonists of apoptosis which are expressed in a wide variety of tissues and tumours which are derived from these tissues. The antigen BCL-2 is part the group of the apoptosis inhibitors [12]. The mode of action for these proteins is the forming of either homodimers or heterodimers. The BCL-2 homodimers and BCL-2/BAX heterodimers inhibit apoptosis [13]. The effects of BCL-2 on clinical course have been widely studied within many lymphoma types in the past. The expression of BCL-2 in DLBCL has been shown to vary between 45% and 55% [14]. The manifestation of BCL-2 in BL has time and again been said to be negative, recent studies have however reviewed that weak expression actually occurs and strong expression is also possible [15]. Others have shown that BCL-2 is expressed in most SLLs, in the majority of FLs and also in low grade mucosal associated lymphoid tissue NHLs. While normal mantle cells have been seen to express high levels of BCL-2, the opposite is seen in MCLs [16]. Virtually all of the small B-cell neoplasms express BCL-2. Overall, approximately 85% to 90% of FLs are BCL-2 positive. BCL-2 expression varies with FL grade with almost 100% expression in grade 1 and approximately 75% in grade 3 [17, 18].

BCL-6

The protein BCL-6 belongs to the Bric-a-brac, tramtrack, broad complex/Pox virus zinc finger (BTB/POZ) zinc finger family of proteins [19]. It mediates transcriptional repression by recruiting co-repressors to an assortment of target genes. The amino side of the BTB domain of BCL6 forms an obligate homodimer, and the interface between BTB monomers forms a specific binding groove for the co-repressors it recruits. The misregulated expression of this factor is strongly implicated in several types of B cell lymphoma [20]. The BCL-6 protein is predominantly expressed in the B-cell lineage where it was found in mature B cells. Immunohistochemical analysis of normal human lymphoid tissues indicated that BCL-6 expression is topographically restricted to germinal centers including all centroblasts and centrocytes [21]. It is also normally expressed in FL and helps separate it from the other small B-cell lymphomas [22].It is

expressed in DLBCL and in BL regardless of the BCL-6 gene rearrangement [21].

Cyclin D1

Cyclin D1 is a protein that is a product of CCND1 gene (located at chromosome 11q13). It is involved in cell cycle progression. Cyclin D1 has been known to interact with cyclin dependent kinase (cdk4 and cdk6) by forming a complex with the enzyme and in that way encouraging proliferation of tumour cells [23]. Translocation involving cyclin D1 and the IgH chain t(11;14)(q13;q32) is present in nearly all MCL. Over expression of Cyclin D1 has been seen in the majority of cases even if the translocation is not detectable. With the rare exception of some cases of hairy cell leukaemia, prolymphoctyic leukaemia, and plasma cell disorders, expression of Cyclin D1 is specific for MCL [24]. Lucioni and colleagues in their study found some CD5 negative DLBCLs expressing Cyclin D1. Hence for the blastoid type of MCL it is important to rule out DLBCL [25]. This study was aimed at detecting the expression of surface marker proteins CD10, CD23, BCL-2, BCL-6 and Cyclin D1 in B-cell NHLs using primary monoclonal antibodies by immunohistochemistry to provide diagnostic and prognostic information on the NHLs presenting in black indigenous Zambians.

Materials and Methods

This was a laboratory based cross-sectional study that was done on archived formalin fixed paraffin embedded tissue (FFPET) found in the histopathology laboratory at the University Teaching Hospital in Lusaka, Zambia. A convenient sample of twenty eight (28) patients' biopsies diagnosed as B-cell NHLs all being positive for both CD45 and CD20 and negative for CD3. These histologically showed features consistent with the following diagnoses; 11 cases of diffuse large B-cell lymphoma (DLBCL), 5 cases of Burkitt lymphoma (BL), 2 cases of Follicular lymphoma (FL), 2 cases of Small lymphocytic lymphoma (SLL) and 8 cases of B-cell NHL unclassified. Using CD10, BCL-2, BCL-6, CD23 and cyclin D1, we further characterized these lymphomas by detection of their respective expression of surface membrane proteins.

Tissue preparation

The sections were first cut using a microtome machine into 4-5µm sections with the blade angled at 4-6°. The cut ribbon was picked by a pair of forceps and transferred onto the water bath set at a temperature of 35-37^{O} C to allow the sections to stretch for a few seconds. The sections were then carefully separated and each section picked on a glass slide at an angle to allow the water to drain off. The glass slides were then placed on a warm plate for about 15 minutes to help the section adhere to the slide. They were then deparaffinised through xylene and dehydrated through alcohol (deparaffinised for 5 minutes twice and four changes of alcohol for 5 minutes each, 2 changes of absolute alcohol, 1 change of 95% alcohol and 1 change of 80% alcohol).

Antigen retrieval

Antigen retrieval was then performed on the specimens using the Envision FLEX Target Retrieval Solution. The solution was pre heated to 95 - 99 °C in an incubator. The sections were immersed in the preheated Retrieval Solution for 20 minutes. Care was taken to ensure the slides were covered by Retrieval Solution throughout the process. The slides were allowed to cool in the Retrieval Solution for 20 minutes at room temperature. The slide rack was removed from the container and the sections rinsed with cold running tap water immediately to prevent drying out of tissue. The rack with rinsed sections was placed in Wash Buffer working solution at room temperature for 5 minutes.

Immunohistochemistry

A humidified staining chamber for the staining procedure steps was prepared to prevent drying out of slides. The slides were first immersed into a 0.3-3% H2O2 and 100% methanol for 5 minutes at room temperature to quench endogenous peroxidase. They were then rinsed in cold wash buffer for 5 minutes. The slides were then placed in the prepared humidifying chamber and a pap pen was used to mark the edges of the tissue on the slide so as to provide a heat-stable, water-repellent barrier that keeps reagents localized on tissue specimens. Drops of the primary antibody were then placed on the tissues on the slides and left in the humidifier for 20 minutes. The antibody was rinsed off from the slides with cold wash buffer for 5 minutes. After that they were placed back in the humidifier and the labelled streptavidin biotin (LSAB) link was then paced generously on the slides and the slides were left in the humidifier for 30 minutes. The slides were again rinsed in wash buffer for five minutes and placed

back in the humidifier and LSAB Streptavidin Peroxidase was poured on the slides and the slides were left in the humidifier for 30 minutes. Thereafter, the slides were again rinsed in cold wash buffer for 5 minutes and then placed in 3, 3'-diaminobenzidine (DAB) working solution for 5 minutes, the DAB gets oxidized in the presence of peroxidase and hydrogen peroxide resulting in the deposition of a brown, alcohol-insoluble precipitate which gives some colour contrast on the slides. The slides were placed in haematoxylin for about 30 seconds to a minute to create a light counterstain. The slides were then placed under running tap water for 5 minutes and they turned blue. The sections were then dehydrated through 3 changes of alcohol (5 minutes each in 80%, 95% and absolute alcohol respectively) and cleared in 2 changes of xylene (3 minutes each).They were cover slipped and the mounting medium used was cryoseal. They were then allowed to air dry and were read on a light microscope and the results were then determined to be either positive or negative for each antibody.

Primary Antibodies used

The following panel of primary antibodies were used: CD10, CD23, CyclinD1, BCL-2 and BCL-6. These antibodies were from Dako, Glostrup, Denmark 2012.

Data analysis: Data was analysed using SPSS version 16.0 software for windows; Univariate analysis of antibody profiles was conducted to determine their distribution patterns of the NHL. Fisher's exact test was used to evaluate statistical significance which was shown by P-value less than 0.05.

Ethical consideration: The study protocol was approved by the University of Zambia biomedical and research ethics committee (UNZABREC). And consent was granted by the university teaching hospital administration. Permission to use the archival histopathological tissues was granted by the senior medical superintendent of the university teaching hospital.

Results

The patients in this study were aged between 4 to 65 years with a mean age of 30.8 years. There was male predominance with a male to female ratio of 2.1: 1. With the age and sex there was no significant difference between the ages and CD10 expression (*P*= 0.42), BCL-2 expression (*P*= 0.20), BCL-6 expression (*P*= 0.60), CD23 (*P*= 1.00) and Cyclin D1 expression (*P*= 0.59) or the sex and CD10 expression (*P*= 0.67), BCL-2 expression (*P*= 0.67), BCL-6 (*P*= 0.60), CD23 expression (1.00) and Cyclin D1 expression (*P*= 0.56).

Immunohistochemistry of the B-cell NHLs

CD10 Expression

The CD10 expression showed no statistically significant results across all the B-cell NHL histological subtypes but BL 4/5 (80%) had the lowest *P*- value of 0.06; for DLBCL 3/11(27.4%, *P*= 0.44), for MCL 1/4 (25%, *P*= 1.00), for FL 1/2(50%, *P*= 0.51), for SLL 1/2 (50%, *P*= 1.00) and for Unclassified B-cell type 3/4 (75%, *P*= 1.00).

BCL-2 Expression

The BCL-2 had similar results with the CD10 showing statistically insignificant results, for DLBCL 6/11 (54%, *P*= 0.20), for MCL 3/4 (75%, *P*= 0.27), SLL 1/2 (50%, *P*= 1.00). The BL, FL and Unclassified B-cell had no expression of BCL-2 with *P*-values of0.62, 0.51 and 0.13 respectively.

BCL-6 Expression

Burkitt lymphoma showed statistically significant results with *P*-value of < 0.0001, the others showed no significant difference in their results with DLBCL 1/11 (9%, *P*= 0.36) and SLL 1/2 (50%, *P*= 1.00). There was no expression of BCl-6 in MCL, FL and Unclassified B-cell NHLs and these finding was of no significance with *P*-values of 0.55, 1.00 and 0.55 respectively.

CD23 Expression

The only subtype that showed expression of CD23 monoclonal antibody was the SLL with 100% expressivity and *P*-value of < 0.0001; the rest had a *P*-value of 1.00.

Cyclin D1 Expression

Similarly to the CD23 antibody, the Cyclin D1 antigen was expressed by one subtype in this case MCL with 100% expression and *P*-value of < 0.0001. The other subtypes did not express this antigen and the finding was statistically insignificant.

Antibody	Clone	Code	Source	Antigen retrieval	Dilution
CD 10	56C6	Is648	Dako	Envision FLEX Target Retrieval Solution	Prediluted
BCL-2	SP4	Is614	Dako	Envision FLEX Target Retrieval Solution	Prediluted
BCL-6	PG-B6p	Is625	Dako	Envision FLEX Target Retrieval Solution	Prediluted
CD23	1B12	Is781	Dako	Envision FLEX Target Retrieval Solution	Prediluted
Cyclin D1	DCS-6	Is083	Dako	Envision FLEX Target Retrieval Solution	Prediluted

Table 1: Antibodies used in the study

Table 2: Expression of membrane surface markers by B-cell NHL subtypes. DLBCL, Diffuse Large B-cell lymphoma; BL, Burkitt Lymphoma; MCL, Mantle Cell Lymphoma; FL, Follicular lymphoma; SLL, Small Lymphocytic Lymphoma; P, P-value; +, Fully expressed; +/-, More expressed; ±, Equally expressed; -/+, Less expressed; -, Not expressed.

Monoclonal Antibodies	B-cell Non-Hodgkin's lymphoma subtypes					
	DLBCL	**BL**	**MCL**	**FL**	**SLL**	**Unclassified**
CD10	-/+ (P = 0.44)	+/- (P = 0.06)	-/+ (P = 1.00)	+ (P = 0.51)	± (P = 1.00)	± (P = 1.00)
BCL-2	+/- (P = 0.25)	-/+ (P = 0.62)	+/- (P = 0.27)	- (P = 0.51)	± (P = 1.00)	- (P = 0.13)
BCL-6	-/+ (P = 0.36)	+/- (P = 0.00)	- (P = 0.55)	- (P = 1.00)	± (P = 0.39)	- (P = 0.55)
CD23	- (P = 0.51)	- (P = 1.00)	- (P = 1.00)	- (P = 1.00)	+ (P = 0.00)	- (P = 1.00)
Cyclin D1	- (P = 0.13)	- (P = 1.00)	+ (P = 0.00)	- (P = 1.00)	- (P = 1.00)	- (P = 1.00)

Figure 1

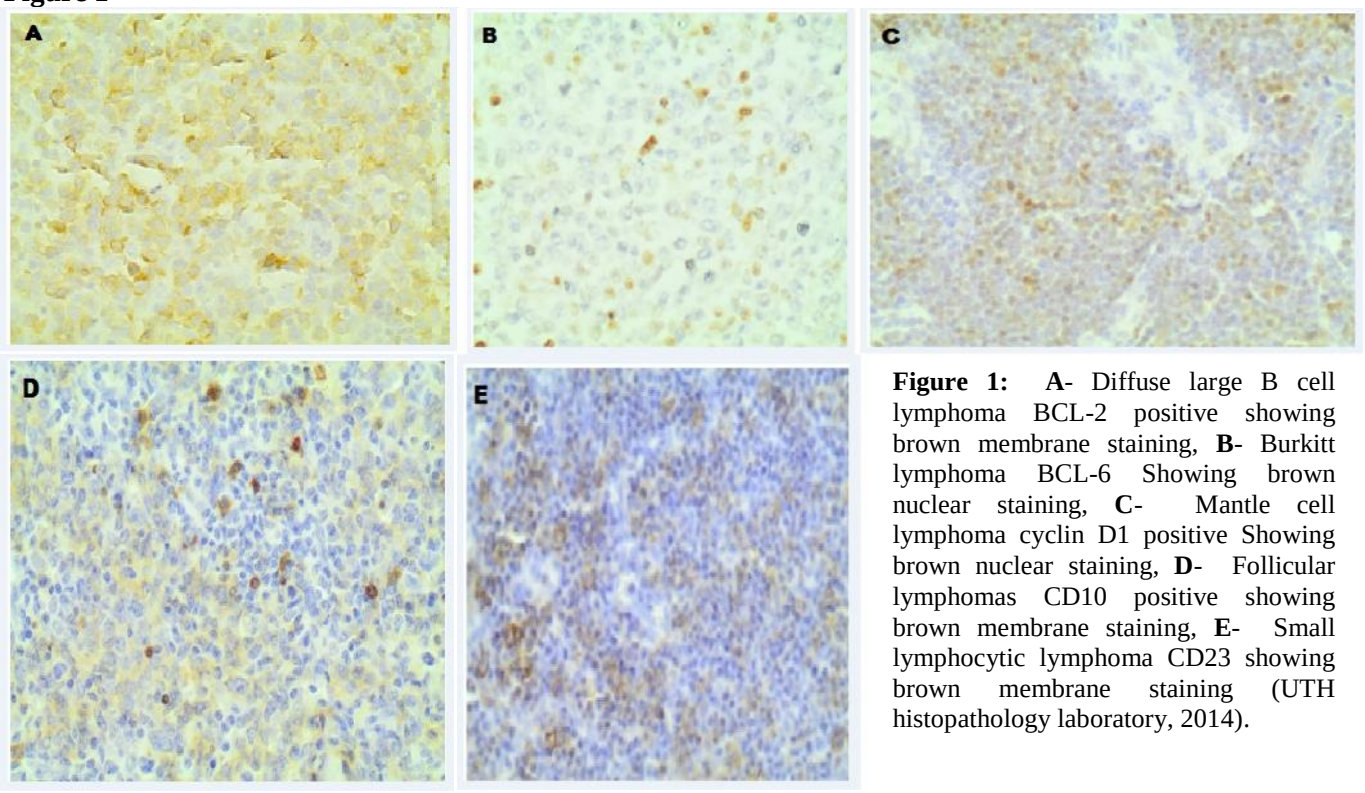

Figure 1: **A**- Diffuse large B cell lymphoma BCL-2 positive showing brown membrane staining, **B**- Burkitt lymphoma BCL-6 Showing brown nuclear staining, **C**- Mantle cell lymphoma cyclin D1 positive Showing brown nuclear staining, **D**- Follicular lymphomas CD10 positive showing brown membrane staining, **E**- Small lymphocytic lymphoma CD23 showing brown membrane staining (UTH histopathology laboratory, 2014).

Discussion

The B-cell NHLs have demonstrated a great amount of heterogeneity in their staining patterns. Hence it is important to use immunohistochemistry to characterise these malignancies as better identification and classification of these B-cell neoplasms may lead to development of subtype specific therapeutic regimens.

By means of gene expression profiling, DLBCL has been placed in three distinct groups; germinal centre (GC) DLBCL, activated B cell (AB) DLBCL, and unclassified DLBCL. These have been associated with prognosis of these lymphomas [26]. These groups have been confirmed by use of immunohistochemistry markers which is more cost efficient and easier to incorporate in routine work. The GC markers are CD 10 and BCL-6 while MUM-1 and CD 138 are the AB markers. GC B-cell pattern expresses CD10 and/or BCL-6 but not activation markers, activated GC B-cell pattern expresses at least one of GC B-cell markers and one of activation markers, or activated non-GC B-cell pattern expresses MUM1/IRF4 and/or CD138 but not GC B-cell markers [27]. The diffuse large B-cell lymphoma (DLBCL) in this study showed variable positivity for CD10, BCL-2 and BCL-6; and negativity for CD23 and Cyclin D1. The use of MUM1 and CD138 would have given better prognostic data for the DLBCL. In previous studies the presences of CD10 and BCL-6 have been associated with good prognosis and better clinical outcome respectively [28, 29]. However only 3 cases (27.3%) showed CD 10 positivity and 1 case (0.09%) showed positivity for BCL-6. This is suggestive of poor outcome for the DLBCL. Another study by Dogan *et al* [30], showed that in DLBCL cases, CD10 positivity was frequently associated to a known history of Follicular lymphoma. Recent studies have shown an association between BCL-2 expression and MYC translocation in DLBCL. Those cases with co-expression of the two are called double hit DLBCLs and have been shown to have a very poor prognosis [31]. BCL-2 was positively expressed in over 50% of the DLBCLs; hence the need to use the MYC protein in DLBCL immunohistochemistry so that if there is co-expression of MYC and BCL-2, aggressive treatment might be implemented.

Burkitt lymphoma tumour cells express B-cell-related antigens and are positive for germinal centre origin antigens CD10 and BCL6, as was shown in this study with positivity of almost 100% percent for both antigens [32]. However it was negative for the BCL-2 which is similar to what has been found in other studies. BCL-2 has been a useful marker in differentiating between reactive and neoplastic B-cell neoplasms, and its expression is mostly in small B-cell neoplasms and in none of his 5 cases of Burkitt lymphoma [18]. Similarly in a paediatric study for Burkitt lymphoma and diffuse large B-cell lymphoma by Hutchison *et al* [33], all the Burkitt lymphoma cases were also negative for BCL-2. One criterion that is used to identify BL is its high proliferative rate that is reflected by an extremely strong and uniform positivity of the Ki-67 antigen [34]. It would have been good to include Ki-67 antigen to determine the proliferation index (PI) of the Burkitt lymphomas as it has been associated with PI in the range of 99% to 100% [35].

Although the small B-cell lymphomas such as Follicular lymphoma, Mantle cell lymphoma, Marginal zone lymphoma and Small lymphocytic lymphoma show major morphologic overlapping, they have been recently shown to be distinct entities with several biologic and clinical differences. CD10 positivity separates most follicular lymphomas from other small B-cell lymphoid neoplasms; CD23 positivity separates small lymphocytic lymphoma/chronic lymphocytic leukaemia and Cyclin D1 positivity separates Mantle cell lymphoma [36].

The MCL could not be morphologically identified but its antibody expression pattern especially reactivity to Cyclin D1 is what allowed for its identification. Jares and co-authors in their research pointed out that the most useful test for MCL is the strong expression of cyclin D1 which separates it from the other Small cell lymphomas [37]. The staining pattern for MCL found in this study is close to the expected expression for MCL [37], apart from one case which was BCL-2 negative. Similar results were found by Mahmoud *et al* [38] in his study were BCL-2 negativity was seen in 83% of the MCLs. Mendez *et al* [39], in his study on BCL-2 expression says that there is usually over expression in MCL with the t (14; 18) translocation. This may imply then that the BCL-2 negativity is due to lack of the translocation, there is need for cytogenetic studies to verify the result. Addition of CD5 antigen would have also consolidated the diagnosis as MCL is mostly positive for CD5. Up to 95% of Follicular lymphomas (FL) contain a chromosomal translocation between chromosomes 14 and 18,

involving immunoglobulin heavy chain (*IGH*) at 14q32 and *BCL2* at 18q21 [40]. As a result of the translocation, FL generally exhibits abnormal *BCL2* expression. The FL in this study was positive only for CD10 monoclonal antibody. It however showed negativity to both BCL-2 and BCL-6 which is not expected [41]. Harris and colleagues in their report of 1997 WHO classification of neoplastic lesions of haematopoietic or lymphoid tissues state that FL can be diagnosed as such if it positively expresses B-cell marker and an indicator for germinal centre derivation such as CD10 or BCL-2 [42]. Hoeller et al [43], in their study also found BCL-2 negative FLs and most cases that were BCL-2 negative lacked the typical t(14;18) translocation but the underlying pathophysiologic conditions of FLs that lack BCL-2 protein expression are largely unknown. A study of FL presenting in the ovaries by Ozsan et al [44] showed high expression of BCL-2 in low grade but late stage FL. Szereday and co-authors in their study found that decrease in expression of BCL-6 was associated with poor prognosis and transformation of FL to DLBCL [45]. In the same vain strong expression of BCL-6 has been associated with longer overall survival and time to treatment failure. The same is true for CD10 expression; however, in this case there is some dependence on the co-expression with BCL-6 and the international prognostic index [46].

The SLL expressivity was as expected with the exception of 2 cases with positivity for CD10 and negativity for BCL-2 respectively [36]. Dong *et al* [47], in his study found CD10 positivity in 10% of the SLL; therefore it is a likely scenario. As for the case with negative BCL-2; an interesting study by Masir *et al* [48] showed that a subset of SLL neoplastic cells tended to lose BCL-2 when they are in cell cycle. They noticed an inverse relationship between Ki-67 and BCL-2, elation in one caused a decrease of the other. Hence it would be good to identify the Ki-67 expression of these particular cases of SLL.

About four cases of the B-cell Non-Hodgkin's lymphomas remained unclassified. Their positivity for CD10 only could not allow us place this group of lymphomas into any group. More antibodies would need to be used to be able to place them in a particular subtype.

Without precise diagnosis, neither significant research projects nor useful patient management can be instituted. Building local capacity to initiate immunohistochemistry with a limited panel of immunostains would be critical for improving diagnostic standards [49]. Improving on the Zambian's health systems proficiency in diagnosing of NHL by use of immunohistochemistry will open greater opportunities to allow for even more advanced methods that will revolutionize the management of these malignancies.

Conclusion

This study has shown that although the B-cell NHLs presented at the University Teaching Hospital have variable expression of the markers used, the majority show poor prognosis by either not being positive for markers associated with good prognosis (CD10 and BCL-6) or overexpression of markers associated with poor prognosis (BCL-2).In this study the DLBCL which accounted for the majority of the B-cell NHLs showed this type of expression which is highly suggestive of very poor prognosis. Hence these findings warrant the need for further studies that combine clinical and laboratory findings to give a holistic picture of these malignancies in indigenous black Zambians.

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Conflicts of interests: None declared

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