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Expression of Ki-67 in B-Cell Non-Hodgkin's Lymphomas of Indigenous Black Zambians

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Abstract

Immunohistochemistry has revolutionized the diagnosis, treatment and management of B-cell Non- Hodgkin's lymphomas (NHL). Proliferation of tumour cells can be used to demonstrate the progression of these malignancies. Immunohistochemically an evaluation can be made using the nuclear antigen Ki-67. This study's objective was to determine the proliferation index (PI) of Ki-67 of B-cell NHLs. This was a cross section study conducted to evaluate 28 B-cell NHLs at the University Teaching Hospital Histopathology laboratory between November 2013 and April 2014. Tissues were cut for immunohistochemical analysis using a microtome. The monoclonal antibody Ki-67 used in the study was from Dako, Glostrup, Denmark. The Labelled Streptavidin Binding (LSAB) staining was used to amplify and view the reaction. The intensity of the reaction was quantified by taking into account the percentage of tumour cells manifesting nuclear colour reactions. Data was analysed using SPSS version 16.0 software for windows; Univariate analysis of the antibody profile was conducted to determine the distribution patterns of the B-cell NHLs. Fisher's exact test with a P-value of less than 0.05 was considered statistically significant. Of all the 28 cases of B-cell Non-Hodgkin's lymphomas in the study 82.1% (23 cases) expressed the Ki-67 antigen with a mean expression percentage of 46.3%. All the Burkitt lymphoma cases (5/5) were positive for the Ki-67 antigen and had the highest values all of which were above 85%. The rest of the subtypes showed variable expression percentages for the Ki-67 antigen. The results reveal that most cases of B-cell Non-Hodgkin's lymphomas express the Ki-67 antigen. The expression intensity of the majority is highly suggestive of aggressive nature of the B-cell Non-Hodgkin's lymphomas irrespective of the subtype. Hence there is need for more aggressive clinical management of the tumour.

Key words: Ki-67, B-cell Non-Hodgkin's lymphoma, Immunohistochemistry.

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Introduction

Non-Hodgkin's lymphomas (NHL) are the most prevalent and diverse haematologic malignancy in the world and the most common type is the B-cell type [1, 2]. The differences in the clinical manifestation has led to classification of NHLs into three categories; indolent, aggressive and very aggressive [3]. This grading is very important as it is used to establish the clinical approach and treatment intensity, but it is very difficult to determine as there is a lot of variation even within individual subtypes [4]. Immunohistochemistry has played a major role in this regard as scientist have identified markers of proliferation that can provide information on the aggressiveness of particular lymphomas early in the diagnostic process. One such marker is Ki-67 protein [4, 5].

Ki-67 protein

Ki-67 is a very large protein which is encoded by about 30, 000 base pair in the human genome and is approximately 395kDa in size [6]. It has a fork head associated (FHA) domain which makes it structurally similar to different proteins that are involved in cell regulation such as kinases, phosphatases and other metabolic enzymes [7]. The Ki-67 protein has been noted to be located in the nucleolus during interphase and

it migrates to the perichromosomal region during mitosis [8]. The exact function of the protein Ki-67 is not well known but its association with the dense fibrillary compartment (DFC) of the nucleoli [9] which contains proteins involved in ribosomal gene transcription has led to the assumption that Ki-67 maybe also involved in the synthesis of ribosomal RNA [10]. The expression of Ki-67 has generally been in proliferating cells and absent in quiescent cells making it suitable for assessment of tumour proliferation as a cell marker [11]. Some studies have however discovered that Ki-67 is also expressed by quiescent cells only that it is strongly down regulated and needs highly sensitive techniques such as chromatin immunoprecipitation (ChIP) assays to be detected. However, using standard histopathological techniques Ki-67 was not recognised in resting cells on paraffin sections, even when highly elevated concentrations of the MIB-1 antibody were used. Hence it remains useful as a marker of proliferation [12]. Though the exact function of Ki-67 is not known, made particularly difficult by its unique structure which prevents derivation of function by comparison, it is important to note that Ki-67 is vital for cell proliferation, which has been shown by halted cell proliferation when Ki-67 is removed from the cell using antisense nucleotides [5, 6].

Clinical significance

The protein Ki-67 has been found in a number of studies to have diagnostic, prognostic and predictive value in different neoplasms. Diagnostic value in being able to distinguish one type of condition from the other, prognostic value in being able to objectively conclude from its expression the likely clinical course a disease can be inferred and predictive value as an indicator of the relative sensitivity (or resistance) of a disease to a particular therapy based on its expression [13].

The protein Ki-67 has enabled the differentiation between benign and malignant lesion; it has been used in separating high-grade squamous intraepithelial lesions tumour cells in cervical or anal lesions which are Ki-67 positive from normal or atrophied cells which are generally negative [14]. It is also being used as a diagnostic clue together with histological features in differentiating between malignant melanoma and other difficult melanocytic lesions. A melanocytic lesion with borderline histological features for malignancy and high pKi67 expression (>5%) would be best interpreted as a melanoma [15, 16]. A lot of studies have been done in relation to Ki-67 and breast cancer where it has shown both prognostic as well as predictive value. High expression of Ki-67 has been associated with reduced overall survival as well as disease free time primary breast carcinomas [17]. The expression of Ki-67 was also seen to independently improve the prediction of treatment response and prognosis in a group of breast cancer patients receiving neoadjuvant treatment [18]. In Non-Hodgkin's lymphoma the Ki67 stain alone is a powerful tool for distinguishing benign from malignant proliferations within the selected groups. It also allows evaluation of the distribution of proliferating cells in addition to simply determining the proliferation rate of cells. [19].

In this study we aimed to detect the expression of the nuclear proteins Ki-67 in B-cell NHLs using a primary monoclonal antibody by immunohistochemistry to provide diagnostic and prognostic information on the NHLs prevailing in indigenous black Zambians.

Materials and Methods

The study design and sampling

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 (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 [20]. These B-cell lymphomas were characterized by detection of their expression of surface proteins Ki-67 using the monoclonal antibody Ki-67.

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 the forceps and transferred to 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 in a warm plate for about 15 minutes to help the section adhere to the slide. They were then deparaffinized through xylene and hydrated 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 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 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 was 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 as in procedure 2.7.4 and this time the mounting medium 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 monoclonal antibody used in this study was a ready to use Ki-67 with clone MIB-1 and code Is626 from Dako, Glostrup, Denmark 2012.

Evaluation of the intensity of IHC reaction

The intensity of Ki-67 antigen expression was conducted using categories which took into account the percentage of cells manifesting nuclear colour reactions: less than 5%, 5-29%, 30-59% and more than 60% (Table 1). Percentage of positive cells was evaluated scoring the brownish-labelled cell nuclei detected after screening of all cell nuclei under the microscope manually. The intensity of the IHC reactions was independently evaluated in coded preparations by two pathologists and the mean values were used in the data analysis. The examining pathologists knew no clinical details related to the respective patient.

Ki-67 Results (%)	**B-cell Non-Hodgkin's lymphoma subtypes**					
	DLBCL	**BL**	**MCL**	**FL**	**SLL**	**Unclassified**
< 5	2 (18.2%)	0 (0%)	0 (0%)	2 (100%)	1 (50%)	0 (0%)
5 - 29	1 (9.1%)	0 (0%)	1 (25%)	0 (0%)	0 (0%)	1 (25%)
30 - 59	4 (36.4%)	0 (0%)	3 (75%)	0 (0%)	1 (50%)	2 (50%)
>60	4 (36.4%)	5 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)
P- value	**1.0**	**0.03**	**0.26**	**0.05**	**0.68**	**1.0**

Table 1: Expression of Ki-67 by B-cell Non-Hodgkin's Lymphomas subtypes

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 (UTH).

Results

General characteristics

The ages of the patients in this study ranged between 4 to 65 years with a mean age of 30.8 years. There were more males than females with a male to female ratio of 2.1: 1. There was no significant difference between the ages and Ki-67 expression (*p*- 0.28) neither was there significance differences between the sex and the Ki-67 expression (*p*- 0.69).

Ki-67 expression

Of the entire B-cell NHLs 78.6% expressed the Ki-67 protein demonstrated by a positive reaction in their nuclei as shown in table 1. Combined the B-cell NHLs had a mean Ki-67 PI of 46.5 with a standard deviation (SD) of 33.3. The percentages of expression however were varied between different subtypes. Using the cut-off of 20% 22 cases (78.6%) were above the mark and 13cases (46.4%) were above the 45% cut-off (Table 2).

Diffuse large B-cell lymphoma (DLBCL)

For the diffuse B-cell subtype 2 cases (18.2%) did not express Ki-67 while the rest showed variable expression; 1 case had an expression between 5 to 29%, 4 cases (36.4%) had an expression of between 30 to 59% and 4 cases (36.4%) had an expression of greater than 60%. This reaction was not statistically significant with *p* value of 1.00 as shown in table 1. The mean for this subtype was 44.9% with a standard deviation of 28.6. About 9 cases (81.2%) above the 20% cut-off and 6 cases (54.5%) went above the 45% cut-off (table 2).

Burkitt lymphoma (BL)

This subtype showed statistically significant results (*p*-0.03) with 100% expression of Ki-67 (5 cases). All the cases showed a nuclei reaction of greater than 60% as shown in table 1. The mean for this subtype was 91.8% with a standard deviation of 2.4. About 5 cases (100%) above the 20% cut-off and 5 cases (100%) went above the 45% cut-off (table 2).

Mantle cell lymphoma (MCL)

In this instance 75% of the cases (3 cases) showed positive reaction ranging between 30 to 59%, while one case showed a negative reaction. The reactions were not statistically significant with a *p*-value of 0.26 as shown in table 1. The mean for this subtype was 29.8% with a standard deviation of 20.1. It had 3 cases (75 %%) above the 20% cut-off and no cases (0%) went above the 45% cut-off (table 2).

Follicular lymphoma

None of the cases expressed Ki-67 and this was not statistically significant with a *p*-value of 0.05 as shown in table 1. The mean for this subtype was 0% and no cases went above the 20% or 45% cut-off (table 2).

B-cell NHL type	Ki-67 PI% Mean ± SD	Ki-67 PI		Ki-67 PI		N= 28 (%)
		< 20% (n=6)	>20% (n=22)	< 45% (n=15)	>45% (n=13)	
DLBCL	44.9 ± 28.6	2	9	5	6	11 (39.3%)
BL	91.8 ± 2.4	0	5	0	5	5 (17.8%)
MCL	29.8 ± 20.1	1	3	4	0	4 (14.3%)
FL	0	2	0	2	0	2 (7.1%)
SLL	17.5 ± 24.7	1	1	2	0	2 (7.1%)
Unclassified	49 ± 28.7	0	4	2	2	4 (14.3%)
Total	46.5 ± 33.3	6	22	15	13	28 (100%)

Table-2: Ki 67 proliferative index (PI) in relation to subtypes of NHL, using cut-off value of 20% and 45%.

Small lymphocytic lymphoma

This subtype showed equal expression with 50% showing a positive reaction while the other half did not express Ki-67. This was not statistically significant with a *p*-value of 0.68 as shown in table 1. The mean for this subtype was 17.5 with a standard deviation of 24.7. It had 1 case (50%) above the 20% cut-off and no cases (0%) above the 45% cut-off (table 2).

Unclassified B-cell Non-Hodgkin's lymphoma

All the four cases were positively reactive to the Ki-67 protein, 1 case showed an expression less than 30%, 2 cases showed an expression of between 30 to 59% and the remaining case showed an expression of

greater than 60%. This was not statistically significant (*p*-1.00) as shown in table 1. The mean for this subtype was 49 with a standard deviation of 28.7. It had 4 (100%) above the 20% cut-off and 2 cases (50%) above the 45% cut-off (table 2).

Discussion

Non- Hodgkin's lymphomas are a diverse group of malignant tumours of the lymphoid system [1]. Proliferative activity of the tumour, measured by expression of Ki-67 antigen has been linked to the tumour proliferation rate [21]. The Ki-67 antigen expression is a recognized prognostic index in various tumours [21]. Among the Non-Hodgkin's lymphomas a very strong correlation between a low Ki67 index (less than 20 per cent) and low grade histology and a high Ki67 index (greater than 20 per cent) and high grade histology was found [22]. Broyde and friends used a cut-off 45% to differentiate between indolent and aggressive lymphomas using their mean Ki-67 PI means [4]. Furthermore, higher Ki-67 PI is associated with a poor prognosis, in both solid and haematological malignancies [23].

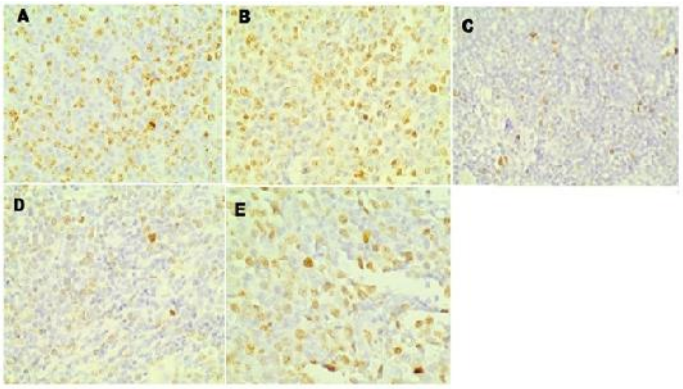

Figure 1: Positive Ki-67 A- Diffuse large B cell lymphoma, B- Burkitt lymphoma, C- Mantle cell lymphoma, D- Small lymphocytic lymphoma, E- Unclassified B-cell Non-Hodgkin lymphoma. All showing brown nuclear staining (UTH histopathology laboratory, 2014).

The Diffuse large B-cell lymphomas (DLBCL) showed varied expression of the Ki-67 antigen. Though more than 50% of them were positive for Ki-67 their staining intensity was spread out revealing a large proliferation index ranging from 26% to 82% similar to what was seen by Frost and friends [24]. The high PIs have also been associated with very poor prognosis for DLBCL patients [25]. High proliferation index (PI) of DLBCL has also been shown in some studies to show co-expression with the protein c-myc and p53 and associated with patients that have high or high-intermediate risk [26] and at a molecular level some of the DLBCLs with high PIs have been seen to be germinal center derived with combined mutation of the BCL2 and MYC genes hence being called double hit lymphomas [27]. In terms of treatment, it was discovered that for non germinal centre DLBCLs with high Ki-67 PIs the survival benefit from the use of Rituximab, Cyclophosphamide, Doxorubicin Hydrochloride (Hydroxydaunomycin), Vincristine Sulfate (Oncovin), Prednisone combination (R-CHOP) which is an effective drug combination is limited [28].

All cases for BL showed strong positivity for Ki-67, with the lowest being 88% and the highest 94%. This is said to be typical for BL whose proliferation rates are usually high approaching 100% [29]. High Ki-67 of almost 100% was thought to be unique to BL [30]. And Ki-67 PIs of 99% or greater strongly support a diagnosis of BL though a small overlap exists with PIs in the 90%-95% range with DLBCL. In this study however though morphologically the lymphomas exhibited BL features, none had PIs at 100%, the highest was 98% [24]. Hence there is need for further studies including molecular analyses which might allow for better classification of these indefinite cases.

For MCL the antibody Ki-67 was positive in all except one case. The Ki-67 index has been confirmed as a very powerful single prognostic factor for overall survival, with highly proliferative cases showing a much poorer outcome than tumours with low proliferation [31]. The poor clinical outcome and the high number of relapses have stimulated research interest for alternative chemotherapeutic approaches for MCL [32].

The protein Ki-67 was negative for all the cases of FL and in the same line Llanos *et al* [33] found low expression of Ki-67 with 96% of the FL in their study showing insignificant expression.

The SLLs in this study showed 50% (1/1) positive expression of the Ki-67 antigen. Masir and co-authors noticed an inverse relationship between Ki-67 and BCL-2 in SLLs, exultation in one caused a decrease of the other. Coincidentally in this study, records reveal that the case that was negative for Ki-67 was positive for BCL-2 and the other case showed a reverse result [34]. Another study found out that a Ki-67 labeling index that exceeds 30% indicated suspected transformation and was strongly suggestive of at least high-grade SLL-Accelerated phase if the lymphoma had not yet transformed fully [35].

For the B-cell groups that could not be placed in any group it was very difficult to determine the

significance of their expression of Ki-67 antigen. Hence more advanced techniques such as fluorescence in situ hybridisation may be needed to better classify these cases.

Conclusion

A significant expression of Ki-67 PIs is seen in general and this is highly suggestive of the aggressive nature of the NHLs irrespective of the histological subtype. Since the Ki-67 is very important in the assessment of indolent and aggressive lymphomas and has both prognostic and therapeutic implications. There is need for further analysis and inclusion of this antibody in the routine diagnostic workout of the patients with B-cell NHLs.

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Conflict of Interest: Authors declare no conflict of interest.

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