ORIGINAL ARTICLE

Expression of Ki-67 in Malignant and Premalignant Cervical Lesions in Nigerian Women

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ABSTRACT

Background: Cervical cancer, though preceded by treatable premalignant lesions, ranks second among all cancers in Nigerian women. The proliferative marker 'Ki-67' is useful immunohistochemically to enhance the diagnosis of cervical dysplastic lesions, reducing inter-and intra-observer variability. This study is aimed at evaluating the role of Ki-67 expression in cervical dysplastic lesions as a diagnostic and prognostic tool.

Methodology: We applied Ki-67 immunohistochemical staining on 142 cervical biopsies from the archives of Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi in Anambra state, a federal teaching hospital. Ki-67stains nuclei of proliferating cells, and was expressed as Ki-67 scores and labeling index (LI). LI was calculated as the number of positive cells per 100 dysplastic cervical epithelial cells while Ki-67 score was given based on levels of positive staining per third of epithelial thickness. The data analysis was done using the IBM SPSS Statistics (Statistical Product and Service Solutions) software version 20.0, and the result presented with tables where relevant.

Results: LI and Ki-67 score increased with increasing dysplasia. There was disagreement between IHC (immunohistochemistry) enhanced and morphologic diagnosis in 9 (6.33%) cases. Ki-67 IHC significantly enhanced the diagnosis of CIN (Cervical intraepithelial neoplasm) and carcinomas (x^2 =0.001, P<0.05). Both premalignant and malignant cervical lesions were more common in fifth and sixth decades.

Conclusion: Ki-67 IHC is a veritable diagnostic and prognostic marker, reducing inter-and intra-observer variability in the diagnosis of cervical dysplastic lesions.

Key words: *Cervical dysplasia; Immunohistochemistry; Ki-67; Labeling index*

INTRODUCTION

While several High Income Countries(HICs) have achieved reasonable success in reducing the burden of cervical cancer, it remains a public health concern among women of reproductive age in Low and Middle Income

Countries(LMICs) including Nigeria.[1] Even more worrisome is the decreasing age of onset of cervical cancer in recent times.[2] Although it is preceded by a detectable and preventable premalignant lesion (a phase of about a period 10 – 15 years),[3] it is still the leading cause of cancer-related deaths in women in LMICs especially the sub-Saharan region including Nigeria.[4,5] Cervical cancer is the second most common malignancy in women worldwide with 500,000 new cases and 250,000 deaths annually.[5,6] In Nigeria, cervical cancer is the second most common malignancy in adult women as well as the most common malignancy of the female genital tract.[7,8]

Cervical carcinomas are preceded by dysplastic alterations in normal squamous differentiation designated as cervical intraepithelial neoplasia (CIN). Both the premalignant and malignant lesions are characterized by increased cellular proliferation and cell cycle abnormalities.[9] Histologic assessment of cervical biopsies for the identification and grading of the cervical lesions is based on identification of deviations from the normal cervical architecture. Grading of these lesions however is fraught with inter and intra-observer variability such that reproducibility may be difficult.[10,11] To minimize this variability, methods for the assessment of cellular proliferation were variously developed, however their use is marred by cost and sophistication.[12]

Antigen Ki-67 is a nuclear non-histone protein encoded by the MKI-67 gene, serving as a biomarker of cell proliferation both in normal and abnormal dividing cells and expressed in all but the G0 phase of the cell cycle.[13] Its expression in normal human cervical squamous epithelium is limited to the proliferating basal and parabasal cells layer, but extends above this layer in the event of dysplasia and carcinoma; the increase in the number of positive cells having a significant positive correlation with ascending grade of CIN.[14] It can therefore be a predictor of the malignant potential and prognosis of lesions.[2,15] Ki-67 labelling index is also noted to increase with increasing grade of dysplasia, hence a determinant of tumour aggressiveness.[16,17] Also, timely monitoring of Ki-67 expression may guide management of cervical dysplastic lesions.[2] Ki-67 immunohistochemical assessment can therefore be used as an added tool to aid correct histologic diagnosis based on the routine Haematoxylin and Eosin (H&E) staining technique, to achieve appropriate diagnosis and treatment. This has been applied widely in the auxiliary diagnosis of malignant and premalignant lesions, especially of the cervix;[18] and can be performed on paraffin-embedded section as an attractive index for prognosis and course prediction.[19]

The aim of this study therefore, is to evaluate the immunohistochemical staining pattern of ki-67 antigen in cervical lesions seen in Nnamdi Azikiwe University Teaching Hospital, Nnewi, a federal teaching hospital in Nigeria.

METHODOLOGY

Study Design, Setting and Ethics

This is a cross-sectional study of archived tissue blocks of cervical lesions from the Histopathology department of Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi. NAUTH is a tertiary teaching hospital located in Anambra state, South-East of Nigeria. An approval was obtained from the Ethics Research Committee of NAUTH before commencement of this research work.

Sampling

A total of 159 cervical biopsies were receive over the study period from January, 2013 to December, 2014, out of which 17 were excluded (8 had missing tissue blocks, 3 tissue blocks were damaged while 6 tissue blocks had inadequate tissue left for sectioning) from the study. Hence, 142 formalin-fixed paraffin embedded cervical specimens were used for this study. Subjects' initial diagnoses were also obtained from clinical records and histopathology reports of the patients. Fresh H&E stained sections were prepared from the retrieved tissue blocks and reviewed blindly by 3 independent pathologists, who were not part of this study, to get a consensus diagnosis.

Immunohistochemistry (IHC) Staining

Avidin biotin Imuunoperoxidase method was used. The immunohistochemical stain for antigen Ki-67 was performed using Novocastra Histology Kit (LEICA, Heidelberg, Germany. Lot: 5713XJL10) Monoclonal antibody to Ki-67 nuclear antigen of mouse origin was used as primary antibody in 1:100 dilution and biotinylated goat antimouse as secondary antibody.

Immunohistochemical studies were done by the Avidin Biotin Complex (ABC) method on the formalin-fixed paraffin-embedded (FFPE) tissue blocks. Four micrometer (4 µm) thick sections of fresh tissue sections were made from the selected FFPE tissue blocks. The tissue sections were de-paraffinized by passing it through xylene and then rehydrated in decreasing alcohol concentrations and mounted on positively charged glass slides. Antigen retrieval was performed by heating the sections on a citric acid solution at PH 6.0 using the microwave at power 100 for 15minutes. The sections were equilibrated gradually with cool water to displace the hot citric acid for at least 5minutes for the section to cool. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Sections were washed with Peroxidase-Blocking Solution (PBS) and protein blocking were performed using avidin for 15min. Sections were washed with PBS and endogenous biotin in tissue was blocked using biotin for 15min, then incubated with the primary antibody, rinsed and then followed by the use of secondary detection system using diaminobenzene (DAB) as chromogen.

Immunohistochemical staining was performed using monoclonal antibody to Ki-67 nuclear antigen of mouse origin in 1:100 dilution and incubated for 60 minutes with a positive tissue control in parallel. All the aforementioned steps were carried out at room temperature.

Interpretation of Result

Ki-67 is normally expressed in the nuclei of proliferating cells, and staining is limited to the basal and parabasal layers of normal cervical tissue. Only nuclear staining is considered, and scored as follows:

0 = Nuclear staining limited to 1-2 layers of basal/parabasal

1+ = Nuclear staining confined to the lower third of the epithelium

2+ = Nuclear staining confined to the lower and middle third of the epithelium

3+ = Nuclear staining greater than the lower two-third of the epithelium

Ki-67 labelling index (LI) was calculated by the number of cells showing positive staining per 100 cervical epithelial cells in separate representative areas of tumour and the mean was calculated. Ki67 labelling index was calculated as follows:

Labelling index (LI) =

$$\frac{\text{No. of cells showing positive staining}}{\text{Total No. of cells}} \times 100$$

The sections stained for Ki-67 proliferation (revealed as nuclear staining) was graded as:[20]

High Grade: >30% positive cells
Moderate Grade: 16%-30% positive cells
Low Grade: ≤15% positive cells.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics (Statistical Product and Service Solutions) software version 20.0 (SPSS Inc., Chicago, IL, USA), and the result presented with tables. Chi-Square was performed to test for association between antigen Ki-67 expression and the various groups of cervical lesions and for differences between the diagnosis assisted by antigen Ki-67 IHC and consensus diagnosis by H&E. A *p*-value of <0.05 was considered statistically significant.

RESULTS

A total of 142 cervical biopsies were reviewed by conventional staining technique (H&E staining method). The specimens were gotten via hysterectomy, curettage and punch biopsies. The consensus morphologic diagnosis categories included 24 benign, 69 premalignant and 49 malignant lesions (see Table 1).

Table 1. Morphologic Categories of cervical lesions

	Frequency	Percent
Malignant		
SCC	35	24.6
Adenocarcinoma	11	7.7
Adenosquamous carcinoma	3	2.1
Sub total	**49**	**34.5**
Premalignant		
CIN I	29	20.4
CIN II	20	14.1
CIN III/CIS	20	14.1
Sub total	**69**	**48.6**
Benign		
Chronic cervicitis	12	8.5
Genital wart	2	1.4
Endocervicitis	2	1.4
Endocervical polyp	7	4.9
Nabothian cyst	1	0.7
Subtotal	**24**	**16.9**
Total	142	100.0

*SCC= Squamous cell carcinoma; CIN/CIS= Cervical intraepithelial neoplasia/Carcinoma in situ

Following the application of Ki-67 immunohistochemical staining, 9 cases of misdiagnosis were noted. These included 4 of the 29 morphologically diagnosed CIN I cases, which were found to be benign and one found to be CIN II (see tables 2a and b).

Table 2a. Comparison between H&E diagnosis and IHC assisted diagnosis

Consensus Diagnosis		**Diagnosis assisted by IHC**						**Chi-square**	***P*-value**
		Benign	CIN1	CIN2	CIN3/CIS	SCC	ADC/ASC		
Benign	24	24(100%)	0	0	0	0	0		
CIN1	29	4(13.8%)	24(82.8%)	1 (3.4%)	0	0	0		
CIN2	20	0	2(10%)	17(85.0%)	1(5.0%)	0	0	476.394	0.001
CIN3/CIS	20	0	0	1(5.0%)	19(95%)	0	0		
SCC	35	0	0	0	0	35(100%)	0		
ADC/ASC	14	0	0	0	0	0	14(100%)		
Total	**142**	**28**	**26**	**19**	**20**	**35**	**14**		

SCC= Squamous cell carcinoma; ADC= Adenocarcinoma; ASC= Adenosquamous carcinoma; CIN/CIS= Cervical intraepithelial neoplasia/Carcinoma in situ; IHC= Immunohistochemistry; H&E= Haematoxylin and eosin.

Table 2b. Descriptive statistics of antigen ki-67 expression for different cervical lesions.

Consensus diagnosis	Epithelial Ki-67 level					Total
	Negative	**1+**	**2+**	**3+**	**4+**	
Benign	24	0	0	0	0	24
CIN I	4	24	1	0	0	29
CIN II	0	2	17	1	0	20
CIN III/CIS	0	0	1	19	0	20
SCC	0	0	0	0	35	35
Adenocarcinoma	11	0	0	0	0	11
Adenosquamous carcinoma	0	0	0	0	3	3
Total	**39**	**26**	**19**	**20**	**38**	**142**

SCC= Squamous cell carcinoma; CIN/CIS= Cervical intraepithelial neoplasia/Carcinoma in situ

The different lesions displayed nuclear Ki-67 positivity limited to specific epithelial level depending on the presence and grade of dysplasia (see table 2b and figures 1A-J)

It was found that the application of antigen Ki-67 IHC enhanced the differential diagnosis of cervical lesions (x^2 =0.001, p=0.001).

Age Distribution of the Cervical Lesions

The mean age of occurrence of the benign cervical lesions was 48.4 ±12.06 years, while that for the premalignant and malignant lesions were 50.8 ±10.98 and 54.0 ±14.06 years, respectively. The premalignant lesions were found to be more (33.9%) in the fifth decade of life (see Table 3a), while 11 (31.4%) of squamous cell carcinoma occurred in the seventh decade (see Table 3b).

The adenocarcinoma group occurred more in a lower age group of fifth decade (54.6%) than the squamous cell carcinoma group (Table 3b). Among the squamous cell carcinoma group, the non-keratinizing variant was accounted for the majority (60.0%) of cases.

Ki-67 Labelling Index for Premalignant and Malignant Cervical Lesions

Twenty-two (88%) of CIN I and 34 of High grade squamous intraepithelial lesion (HSIL) including 17 (89.5%) of CIN II and 17 (89.5%) of CIN III express low, moderate and high Ki-67 labelling index respectively. There is increase in the Ki-67 labelling index with increasing grade of the lesions (x^2 =126.349, P<0.001) (see Table 4).

All 3 (100%) cases of adenosquamous carcinoma and 24 (60.0%) of the SCC group, including mainly the basaloid and high grade non-keratinizing variants had high Ki-67 labeling index (see table 4b)

Table 3a. Age-group distribution of cervical lesions categories

Age group of patients	Biologic behaviour		Total
	Malignant	**Premalignant**	
21-30	1	1	2
31-40	5	10	15
41-50	16	22	38
51-60	11	20	31
61-70	11	9	20
71-80	2	3	5
>80	3	0	3
Total	49	65	114

Table 3b. Age group distribution of the premalignant and malignant cervical lesions

Age Grouping	Ki-67 Enhanced diagnosis						
	CIN I	CIN II	CIN III	SCC	ADC	ASC	Total
21-30	1	0	0	0	0	1	2
31-40	4	4	2	4	1	0	15
41-50	9	6	7	10	6	0	38
51-60	10	3	7	7	3	1	31
61-70	2	4	3	11	0	0	20
71-80	0	2	1	1	0	1	5
>80	0	0	0	2	1	0	3
Total	26	19	20	35	11	3	114

ADC= Adenocarcinoma; ASC= Adenosquamous carcinoma; CIN= Cervical intraepithelial neoplasia; SCC= Squamous cell carcinoma

Table 4. Assessment of Ki-67 labelling indices of the premalignant and malignant cervical lesions.

		Ki-67 level Index			**Total**	**Chi-square**	***P*- value**
		Low	**Moderate**	**High**			
Ki-67 Enhanced diagnosis	ADC	1	6	4	11		
	ASC	0	0	3	3		
	CIN I	23	3	0	26		
	CIN II	1	18	1	20	126.349	<0.001
	CIN III	0	2	17	19		
	SCC	0	11	24	35		
Total		**25**	**40**	**49**	**114**		

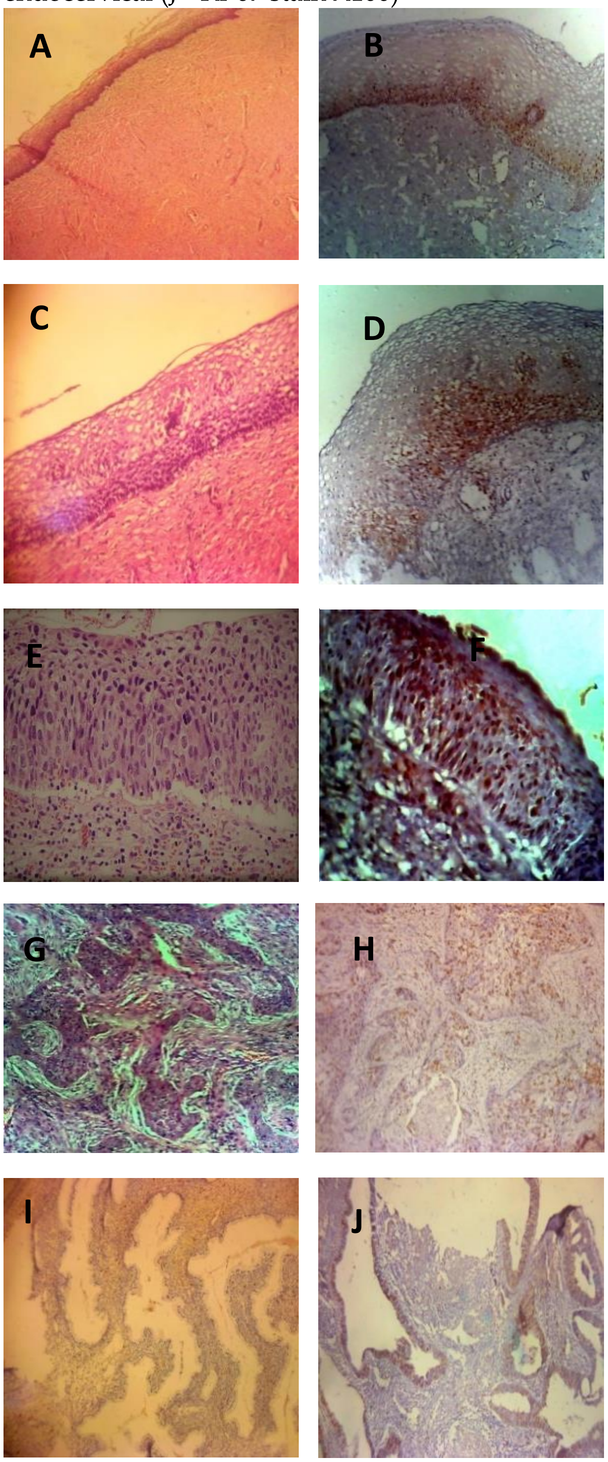
ADC= Adenocarcinoma; ASC= Adenosquamous carcinoma; CIN= Cervical intraepithelial neoplasia; SCC= Squamous cell carcinoma

Table 4b. Ki-67 labelling indices of the SCC variants

		Ki-67 labeling Index			**Total**
		low	**moderate**	**high**	
SCC Variant	LCK	0	6	8	14
	LCNK	0	5	11	16
	Basaloid	0	0	5	5
Total		**0**	**11**	**24**	**35**

LCK= Large Cell Keratinizing; LCNK= Large Cell Non-Keratinizing

Figures 1A-J. Showing normal and different epithelial dysplastic lesions of the cervix. Normal ectocervix (A=H&E X50, B=Ki-67 stain X100); LSIL/CIN1 (C=H&E X100, D= Ki-67 stain X100); HSIL (E= H&E X100, F= Ki-67 X100); SCC (G=H&E X100, H= Ki-67 stain X100); Normal endocervix (I= Ki-67 stain X100); Dysplastic endocervical (J= Ki-67 stain X100)



DISCUSSION

The basic element of the lesion and its precursors is uncontrolled cell proliferation. The Low grade (CIN I) and/or high-grade (CIN II and CIN III) precursor lesions are known to either regress or progress to invasive cervical carcinoma after a period of time, influenced by the persistence of Human Papilloma Virus (HPV) infection.[16,21] Early detection of HPV infection and these precursor lesions are methods for the identification of women at risk of developing cervical cancer. This however, is fraught with inter- and intra- observer variability.[22] Assessment using some biomarkers, including Ki-67 proliferation marker helps proper diagnosis and grading of these lesions.

The observation in this study was that there were 13.8% misdiagnosis/misclassification using H&E morphology alone (see table 2a). Ki-67 immunohistochemical staining therefore enhanced accurate diagnosis/classification of the cervical lesions, preventing under treatment/overtreatment (p<0.001). This is similar to the Netherland work of Bulten *et al.* which reported that some cases of morphologically diagnosed high grade lesions were found to be normal atrophic changes following Ki-67 immunohistochemistry.[23] Son *et al.* also reported the utility of Ki-67 immunohistochemical staining in diagnosing cervical lesions, as they employed it to differentiate squamous metaplasia and normal cervical mucosa (which showed negative expression) from squamous cell carcinoma and CINs of the cervix which expressed increased staining.[24] Wright *et al.* concluded that Ki-67 staining is a useful tool in reducing inter-observer variability in diagnosing cervical dysplastic lesions.[25]

Apart from enhancing differentiation between dysplastic and non-dysplastic cervical lesion, studies have shown that Ki-67 can be used in stratifying the different grades of dysplasia, as well as for a risk assessment of detected lesions, predicting progression, and to monitor recurrences after treatment.[14,26] This index study is in agreement with several studies which demonstrated increasing epithelial level of Ki-67 positivity with increasing grades of CINs. We also noted an increase in Ki-67 labelling index proportionate to increasing grades of dysplasia (x^2 =126.349, p<0.001). Ki-67 index was high for all (100%) adenosquamous carcinoma compared to the squamous cell carcinoma group which showed high Ki-67 index in only 68.6% of the cases, with majority of the high index SCC cases being the high grade non-keratinizing and basaloid variants which have poorer prognosis than the well differentiated keratinizing variant. Milana *et al*. in agreement, reported a positive correlation between level of distribution of Ki-67 positive cells and CIN grades.[27] Ki-67 is therefore a valid tool not only for diagnosis, but also for predicting progression of CINs to invasive carcinoma, as well as to prognosticate dysplastic lesions.

A meta-analysis of 13 journals which involved 894 patients by Reza *et al*. gave credence to the above, concluding that Ki-67/MIB1 is a prognostic marker in cervical cancer. Their finding was that patients with high Ki-67/MIB1 expression had significantly less overall survival than patients with low expression of Ki-67/MIB1 (p<0.001).[28] Anju *et al*. stated moreover, that Ki-67 protein could serve as a sensitive biological indicator of the proliferative activity and progressive potential of normal, dysplastic and neoplastic cervical changes independent of age and menopausal status especially when HPV infection assessment is missing, and may have some certain therapeutic implications.[29]

In this study, most of the premalignant (33.84%) and malignant (32.65%) cervical lesions occurred in the fifth decade, although the mean ages of occurrence were found to increase from the benign (48.4 ±12.06 years) to premalignant (50.8 ±10.98 years) and malignant (54.0 ±14.06 years) lesions. This is in agreement with a study done in Calabar by Ebughe *et al*. which reported that majority (38.5%) of cervical cancers occurred in the fifth decade with a mean age of 44.6years.[30] Another study in Warri, southern part of Nigeria reported a similar trend, with most (27.5%) of the cervical cancer cases occurring between ages 40-49years.[31] A study in Lagos, Nigeria by Faduyile *et al*., reported 2 peaks of malignancy including the fifth and seventh decades, accounting for 22.1% each and premalignant lesion being commoner in the fifth decade (45.5%).[32] Studies in India reported similar trends, with maximum cases of premalignant and malignant cervical lesions being found in the age group of 40-49 years.[33,34]

In this index study, SCC accounted for majority (71.4%) of the malignant cervical lesions followed by the adenocarcinoma group (22.4%). Among the SCC group, large cell non-keratinizing variant predominates, accounting for 45.7% of the cases. Similarly, Igho *et al.* reported that squamous cell carcinoma was the commonest (79.8%) malignant cervical lesion, 60 (67%) of which were non-keratinizing; ASC and ADC accounted for 5.5%(6) and 6.4%(7) respectively.[31]

CONCLUSION

Ki-67 immunohistochemistry is a veritable marker to enhance the diagnosis of cervical dysplastic lesions, reducing inter-and intra-observer variability; the index being significantly proportional to the grade of lesion. It can therefore serve also as a prognostic indicator. Both the premalignant and malignant lesions were found to occur commonly in the fifth decade of life. Most of the malignant lesions were squamous cell carcinoma, greater percentage of which is the non-keratinizing variant.

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