Immunoexpression of p16^{INK4a} and Ki- 67 Patterns on Cell Blocks to Identify Significant Preneoplastic Cervical Lesions

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Cervical cancer can be prevented by regular screening. The widely used primary screening test is Pap cytology but it has low single test sensitivity and may generate equivocal results. Therefore, new biomarkers are particularly attractive tests that can be used to specifically identify at one visit. The study was aimed to detect the expression of p16^{INK4a} and Ki-67 patterns on cell blocks to identify significant preneoplastic cervical lesions for detection of cervical cancer. A retrospective cross-sectional descriptive study was carried out in HIV infected women attending Antiretroviral Therapy Clinic, Insein General Hospital from July, 2018 to September, 2019. The 50 residual samples of abnormal cervical cytology were recruited. These samples included (38/50) of Atypical Squamous Cell of Undetermined Significance (ASCUS), (6/50) of Low Grade Squamous Intraepithelial Lesion (LSIL), (3/50) of High Grade Squamous Intraepithelial lesion (HSIL), (2/50) of Atypical Squamous Cell but cannot exclude HSIL (ASC-H) and (1/50) of Atypical Glandular Cell (AGC). Their mean age was (33; ±7 years). Cell blocks were prepared from residual cellular sediments of samples. Then, they were stained with H& E and with p16^{INK4a} and Ki-67 immunostaining. In 60% (30/50 cases), the diagnosis made on using cell block were abnormal cervical lesions which were agreement with previous Pap cytology diagnosis ($p<0.001$). According to previous Pap cytology diagnosis, 8% (3/38) of ASCUS, 33% (2/6) of LSIL and 66% (2/3) of HSIL were p16 ^{INK4a} and Ki-67 positive. According to cytomorphologic diagnosis on cell block, 13% (3/24) of ASCUS, 50% (2/4) of LSIL and 100% (2/2) of HSIL were p16 ^{INK4a} and Ki-67 positive. The immunoexpression of p16 ^{INK4a} and Ki-67 exhibited a statistically significant association ($p<0.001$) with the presence of significant pre-neoplastic lesions on cell block. Therefore, this immuno-expression is useful to find out early diagnosis of cancer especially in HSIL/Cervical Intraepithelial Neoplasia (CIN2+). This study high-lighted the use of p16^{INK4a} and Ki-67 on cell block preparation enhances ability to distinguish high grade lesions from less significant conditions in diagnostic ambiguity. The application of this immunostaining will reduce the unnecessary referral for colposcopy and tissue biopsy as well as over and under diagnosis in cytology screening.

Keywords: Cervical cancer, Cell block, p16^{INK4a} and Ki-67 immunoexpression

INTRODUCTION

Cervical cancer exists as a continuing major public health concern in middle-aged women, particularly in resource poor countries. This cancer was the fourth most common cancer in women ranking after breast cancer, colorectal cancer and lung cancer.[1] The long term

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persistence of human papilloma virus (HPV) infection is the main cause in triggering development of cervical cancer. Although the HPV is usually acquired when women become sexually active, the vast majority of women can clear this virus through their immunity. However, persistence of HPV infection may progress to premalignant cervical intraepithelial neoplasia which precedes the development of cervical cancer after several years. Therefore, early detection of premalignant changes is very effective way to reduce the cervical cancer. Nowadays, cervical cancer can be prevented by regular cytological screening, HPV tests and HPV vaccination.

Cervical cytology test has been used as a screening tool for detection of cervical cancer in women. It could be significantly reduced morbidity and mortality from cervical cancer in women. But the sensitivity and specificity of single cytology test for the detection of HSIL/ CIN2+ is unsatisfactorily low[2, 3] and may provide equivocal results which need further work-up to get definite diagnosis. Therefore, the dual stain biomarkers are investigated their accuracy in determining the presence HSIL/ CIN2+lesions on histology. One of the studies showed the sensitivity and specificity of dual stain in detecting histology proven HSIL/ CIN2+was 93.7% and 76.5% while HPV testing was 85.7% and 14.7%, respectively.[4] In 2014, FDA approved the use of HPV test (Cobas, HPV test) for primary cervical cancer screening for women aged 25 years and above. However, though a negative HPV result can almost exclude that women have precancer or cancer, 80-90% of women who tested HPV positive will not have concurrent disease.[5] HPV testing provides sensitivity for HSIL/ CIN2+, but specificity of screening women is limited because most HPV infections are transient and only a low proportion persists and may progress into transforming cancer cells. In this condition, new biomarkers are particularly attractive tests that can be used to specifically identify at one visit those women that need treatment and may reduce the lost to follow up rate.[6] Therefore, dual immunoexpression ($p16^{INK4a}$ and Ki-67) may be particular interest as a screening adjunct or primary screening tool.

$p16^{INK4a}$ is a cyclin dependent inhibitor that has been proven to be strongly over expressed in transforming infections with oncogenic HPV and is believed to be a surrogate marker for precancerous cervical lesions. Detection of overexpression of $p16^{INK4a}$, a biomarker of transforming HPV infections and precancerous cervical lesions, has been shown to be an efficient tool in managing patients with atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesion (LSIL) cytology results, and for triaging HPV-positive women. Ki-67 is a nuclear antigen and cellular proliferation markers expressed in all cell cycle except G_0 phase, which is also overexpressed in HSIL/ CIN2+. In normal cells, the expression of $p16^{INK4a}$ and Ki-67 is mutually exclusive. It is thought that simultaneous detection of $p16^{INK4a}$ overexpression and Ki-67 within a cell would be indicative of deregulation of the cell cycle and transforming HPV infection which may progress to cancer.[7]

Cell block preparation is useful for the detection and diagnosis of many other lesions. It should be complemented to the Pap test, especially for the patients who are positive for diagnostically difficult cases. Examples of difficult morphologic diagnoses include differentiating atypical immature squamous metaplasia from high grade dysplasia, reactive endocervical cells from glandular dysplasia, and squamous from glandular differentiation in high grade lesions.

Moreover, the ability to prepare cell blocks from residual fluid offers a clear technical advantage if ancillary studies are to be performed. The advantages of cell block preparations include the capacity to demonstrate histologic features, the utility of immunostaining and molecular diagnostic techniques and the ability to examine the cells remaining in residual fluid.[8] The application of cytology combined with cell block preparations may improve the diagnostic accuracy of cytology.

General objective:

- To determine the immunoexpression of $p16^{INK4a}$ and Ki-67 patterns on cell blocks to identify significant pre-neoplastic cervical lesions

Specific objectives:

- To determine the immunoexpression of $p16^{INK4a}$ and Ki-67 patterns on cell blocks to identify significant preneoplastic cervical lesions
- To identify the cytomorphologic features of abnormal cervical lesions on cell blocks
- To find out association between abnormal cervical lesions and $p16^{INK4a}$ and Ki-67 immunoexpression on cell blocks

MATERIALS AND METHODS

A retrospective cross-sectional descriptive study was carried out from July, 2018 to September, 2019. After getting informed consent, HIV infected women who were attending Antiretroviral Therapy Clinic, Insein General Hospital has been chosen for the study. Their age range was between 18-48 years. Pap smear samples were collected by using cytobrush and stored in -20°C for long-term storage. The 50 residual samples of abnormal cervical cytology were involved in the study.

The selected cases were (38/50) of Atypical Squamous Cell of Undetermined Significance (ASCUS), (6/50) of Low Grade Squamous Intraepithelial Lesion (LSIL), (3/50) of High Grade Squamous Intraepithelial lesion (HSIL), (2/50) of Atypical Squamous Cell but cannot exclude HSIL (ASC-H) and (1/50) of Atypical Glandular Cell (AGC). Then, cell blocks were prepared from the residual cellular sediment of vials. For cell block preparation, liquid cell suspension was centrifuged in 50 ml tubes for 5 minutes at 400 *g*. The supernatant fluid was decanted or pipetted without disturbing the cell button. The sediment was washed by 5 ml normal saline followed by centrifugation for a second time. Then, 1 ml plasma was added to cell button, and cells were mixed thoroughly with the plasma. Thrombin was added by drop for each cell block. Specimens were mixed gently by rotating the centrifuge tube and then were set aside until a clot formed (generally after 5 minutes). The specimen was fixed in 10% neutral buffered formalin for 10 minutes. Then, it was transferred to a labeled tissue cassette and was submitted for histologic processing. Tissue sections were stained with haematoxylin and eosin (H&E) for cytomorphologic evaluation and $p16^{INK4a}$ and Ki-67 for evaluation of immunoexpression.

Positive immunoexpression of $p16^{INK4a}$ and Ki-67 confirmed that HPV transformed cell progress to cervical cancer. These procedures were performed at Pathology Research Division, Department of Medical Research.

Ethical consideration

The ethical approval was obtained from Institutional Review Board (IRB) Department of Medical Research.

RESULTS

Of the 50 cell blocks studied, the diagnosis made by cytomorphologic features were as follows: Negative for Intraepithelial lesions or malignancy (NILM) in 20 specimens, ASCUS in 24 specimens, LSIL in 4 specimens and HSIL in 2 specimens. Their mean age was (33; ±7 years) and their basic demographic data was shown in Table 1.

In 60%(30/50 cases), the diagnosis made on using cell block were abnormal cervical lesions which were agreement with previous Pap cytology diagnosis ($p<0.001$) as shown in Table 2. The overall findings of $p16^{INK4a}$ and Ki-67 positive immunoexpression cases were 14% (7/50) of these cell blocks, in which ASCUS was 6% (3/50), LSIL was 4% (2/50) and HSIL was 4% (2/50), respectively. According to previous Pap cytology diagnosis, 8% (3/38) of ASCUS, 33% (2/6) of LSIL and 66% (2/3) of HSIL were $p16^{INK4a}$ and Ki-67 positive but there was no expression in ASC-H and AGC.

According to cytomorphologic diagnosis on cell block, 13% (3/24) of ASCUS, 50% (2/4) of LSIL and 100% (2/2) of HSIL were $p16^{INK4a}$

Table 1. Baseline demographic characteristics of women included in this study by their p16^{INK4a} and Ki-67 immunoexpression status

Characteristics	p16 ^{INK4a} /Ki-67		P value
	Positive (n=7)	Negative (n=43)	
	n (%)	n (%)	
Age group (years)			0.23
<=30	1(14.3)	16 (37.2)	
>30	6(85.7)	27(62.8)	
Educational Level			0.13
Illiterate	0	7(16.3)	
Primary	3(42.9)	6(14)	
Secondary	3(42.9)	15(34.9)	
High school	0	13(30.2)	
Graduate	1(14.3)	2(4.7)	
Life time sexual partner			0.54
<2	5(71.4)	35(81.4)	
>=2	2(28.6)	8(18.6)	
Menarche (years)			0.68
<=16	6(85.7)	39(90.7)	
>16	1(14.3)	4(9.3)	
Age at first intercourse (years)			0.5
<=18	3(42.9)	13(30.2)	
>18	4(57.1)	30(69.8)	
Parity			0.86
<=2	4(57.1)	26(60.5)	
>2	3(42.9)	17(39.5)	
Contraception			0.89
No	6(85.7)	36(83.7)	
Yes	1(14.3)	7(16.3)	
Vaginal infection			0.77
Candida albican	2(28.6)	8(18.6)	
Neisseria gonorrhoea	0	1(2.3)	
Others	5(71.4)	34(79.1)	
Speculoscopy			0.08
Cervical erosion	2(28.6)	2(4.7)	
Bleed on Touch	0	3(7)	
Normal	5(71.4)	38(88.3)	
HIV status of spouse			0.11
Unknown	3(42.9)	31(72.1)	
Positive	4(47.1)	9(20.9)	
Negative	0	3(7.0)	
Duration of HIV (years)			0.92
<= 2	2(28.6)	13(30.2)	
>2	5(71.4)	30(69.8)	
CD4 count			0.98
<=350 cells/mm^3	6(85.7)	37(86)	
>350 cells/mm^3	1(14.3)	6(14)	

n=Number

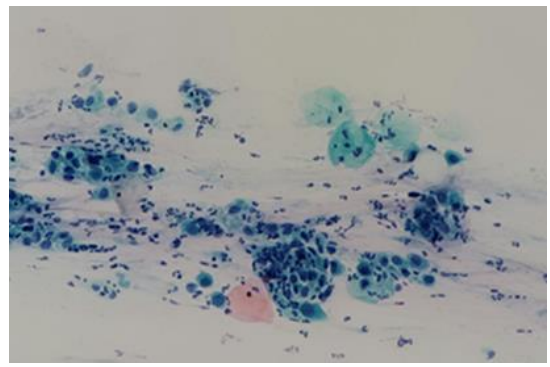
and Ki-67 positive (Table 3). The immunoexpression of cell block for p16 ^{INK4a} and Ki-67 exhibited a statistically significant association (p<0.001) with the presence of significant lesions on cell block.

Table 2. Pap cytology diagnosis and cell block diagnosis

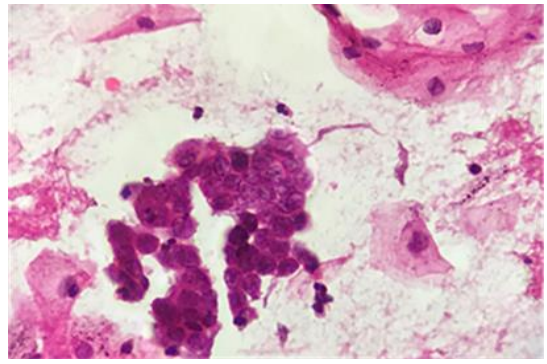
Pap cytology diagnosis	Cell block diagnosis				Total n (%)	P value
	Normal	Abnormal cervical lesions				
	NILM n (%)	ASCUS n (%)	LSIL n (%)	HSIL n (%)		
AGC	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	<0.001
ASC-H	2 (100)	0 (0)	0 (0)	0 (0)	2 (100)	
ASCUS	14 (37)	24 (63)	0 (0)	0 (0)	38 (100)	
LSIL	2 (33)	0 (0)	4 (67)	0 (0)	6 (100)	
HSIL	1 (33)	0 (0)	0 (0)	2 (67)	3 (100)	
Total	20 (40)	24 (48)	4 (8)	2 (4)	50 (100)	

Table 3. p16^{INK4a} and Ki-67 immunoexpression in cell block

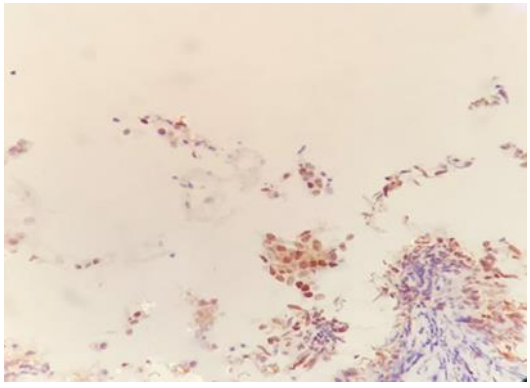
P16^{INK4A}/Ki-67 Immunoexpression	ASCUS n (%)	LSIL n (%)	HSIL n (%)	NILM n (%)	Total n (%)	P value
Positive	3 (13)	2 (50)	2 (100)	0 (0)	7 (14)	<0.001
Negative	21 (87)	2 (50)	0 (0)	20 (100)	43 (86)	
Total	24 (100)	4 (100)	2 (100)	20 (100)	50 (100)	



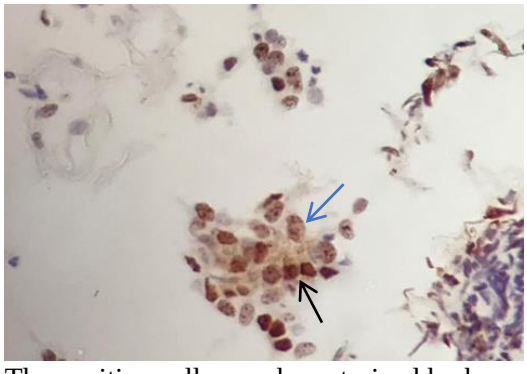
(a) HSIL by Pap stain in pap smear



(b) HSIL by H & E stain in cell block

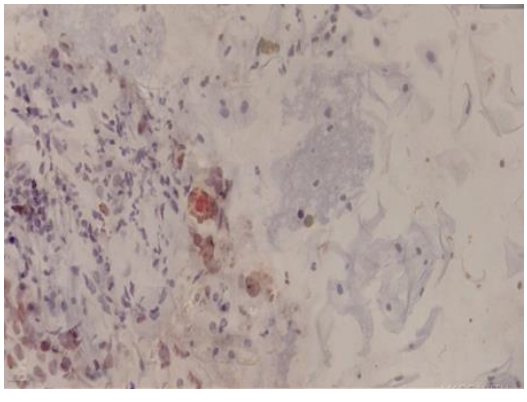


(c-1) $p16^{INK4a}$/Ki-67 Positive Immuno-expression in HSIL (10x)

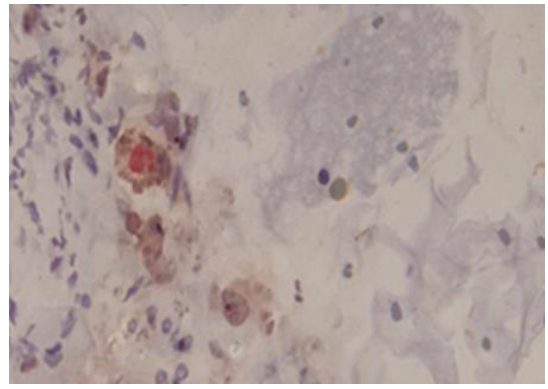


The positive cells are characterized by brown cytoplasmic signal for $p16^{INK4a}$ over-expression and red nuclear signal for Ki-67 expression (Black arrow). Brown cytoplasm and dark red nuclear signals reflected $p16^{INK4a}$ and Ki-67 co-expression (Blue arrow) in same cell

(c-2) $p16^{INK4a}$/Ki-67 Positive Immuno-expression in HSIL (40x)



(d-1) $p16^{INK4a}$/Ki-67 Positive Immuno-expression in LSIL (10x)



(d-2) $p16^{INK4a}$/Ki-67 Positive Immuno-expression in LSIL (40x)

Fig. 1. (a) HSIL by Pap stain in pap smear
(b) HSIL by H & E stain in cell block
(c-1 & 2) $p16^{INK4a}$/Ki-67 Positive Immunoexpression in HSIL
(d-1 & 2) $p16^{INK4a}$/Ki-67 Positive Immunoexpression in LSIL

The features of positive immunoexpression can be detected as in (Fig. 1). The brown cytoplasm signal displayed by $p16^{INK4a}$ staining alone, and the red nuclear signal displayed by Ki-67 staining alone. Positive dual staining cells had brown cytoplasm and dark red nucleus reflected the co-localization of $p16^{INK4a}$ and Ki-67 in the same cell.

DISCUSSION

p16 ^{INK4a} and Ki-67 is a surrogate marker of human papilloma virus infection and cell proliferation, respectively. Over expression of $p16^{INK4a}$ can be used as a biomarker for precancerous and cancerous cervical lesions. Ki-67 can be detected within the nucleus of proliferating cells.

Out of 50 cases, 30 cases (60%) of cell block diagnosis were abnormal cervical lesions which were agreed with previous Pap cytology diagnosis in this study. In one of the study, 73% (62/85 cases) of the diagnosis made using cell blocks were in agreement with the cytology diagnosis.[8]

The other study exhibited that 86 out of 125 of the cell blocks (69%) were found to agree with the cytology diagnosis.[9] There is a little bit variation of findings between studies. This may be due to difference in cytology method. The other studies used liquid based cytology method whereas; conventional method for cytology diagnosis was used in the present study.

The appearance in the cell block may be altered from the smear slightly, due to formalin fixation and difference between routine H&E stain and Pap method. Moreover, it may be due to the lack of diagnostic cells in the residual material from which the cell blocks were prepared. However, cell block has many advantages such as capacity to demonstrate histologic features, feasibility of immunohistochemical stain and molecular diagnostic technique and, no need to do further steps for storage of cells.

In a study of Ebisch, *et al*, altogether 247 cases including both Negative for Intraepithelial Lesion or Maglincy (NILM) and abnormal cervical lesions were tested for p16 ^{INK4a} and Ki-67 immunoexpression and the study found that 55% (136/247) was positive for these marker. In which, 13 out of 88 cases of NILM, 82 out of 91 cases of HSIL and 41 out of 68 cases of ASCUS and LSIL were included of positive immunoexpression.[10]

In the present study, there was no positive immunoexpression in NILM and 14% (7/50) was positive for these markers. This different finding may be due to small sample size of the study. Moreover, small sample size of LSIL and HSIL cases was one of the limitations in the present study. Our study agreed with a study of Korolczuk, *et al*, in which all of the HSIL showed positive immunoexpression.[11] Both studies revealed that HSIL group has high affinity of these markers. This study proved that p16^{INK4a} and Ki-67 immuno-expression was useful in confirmation of precancerous lesions in screening cases and it has high affinity on high grade cervical lesions (CIN2+/HSIL).

Conclusion

Although cell block preparation has extra cost and lengthened specimen turnaround time, the application of cytology screening with cell block may improve the diagnostic accuracy of cytology and other ancillary tests can be performed from these blocks.

This study highlighted the use of p16^{INK4a} and Ki-67 on cell block preparation enhances pathologists' ability to distinguish high grade lesions from less significant conditions and in diagnostic ambiguity. The application of these biomarkers will reduce the unnecessary referral for colposcopy and tissue biopsy as well as false-positive and false-negative results in cytology screening.

In cervical cytology cancer screening, these markers should be an additional test. If there is no chance to perform HPV test, these biomarkers could help to a certain extent to determine further management with the patient.

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