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THE UTILITY OF P16INK4A STAINING ON CELL BLOCKS PREPARED FROM RESIDUAL THIN- LAYER CERVICOVAGINAL MATERIAL

Pathology

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ABSTRACT

Background: Cervical carcinoma is the third most common malignancy in women worldwide accounting for 7.9% of all malignancies. Cervical cytology has become an important tool to screen neoplastic and preneoplastic cases. **Material and Methods:** Our aim of study was to investigate the feasibility and the role of cell block preparations in identifying significant neoplastic and preneoplastic lesions of the uterine cervix and to assess the feasibility of using p16INK4a immunohistochemical staining patterns on cell blocks to identify significant preneoplastic cervical lesions.The cell blocks were prepared from residual material from liquid based cytology of cervical brushings from women with positive cytopathological interpretation of squamous epithelial lesions (i.e ASCUS and above). P-16 immunoreactivity was evaluated in cell block sections in 50 cases (40 cases with positive cytopathology diagnosis + 10 cases which are negative for intraepithelial lesion or malignancy i.e. NILM) with adequate cellularity on a cell block. **Results:** Out of 50 samples, ASCUS (45%) was the most common epithelial cell abnormality followed by LSIL (40%). There were 72% concordant diagnoses on cell block and cytological analysis. Immunostaining of p16INK4a on cell blocks exhibited a statistically significant association ($p<0.05$)) with the presence of significant lesions on either cell block or cytological analysis. **Conclusion:** Cytological smears when supplemented with cell block preparation and application of p-16INK4a on cell blocks can help in better differentiation of high grade, low grade and non neoplastic lesions in case of diagnosis ambiguity and can lead to better triaging of patients reducing patient recall along with decreased non indicated invasive procedures.

KEYWORDS

Cervical cancer, HPV, LBC, Cell block, P-16

INTRODUCTION

Cervical carcinoma is the third most common malignancy in women worldwide accounting for 7.9% of all malignancies, also the second most common malignancy in women in India. It is associated with agonizing morbidity and high mortality.[1] Cervical carcinoma is, however, a preventable disease. Early detection and treatment are the key to reduction in morbidity and mortality due to cancer. Screening for carcinoma cervix has been proved to be beneficial because of the natural history of the disease which allows it to be detected at the preinvasive stage of dysplasia or carcinoma in situ . This lead to a decrease in mortality by more than 70% since cervical cancer screening became widely available in the United States. The sensitivity of a conventional pap smear is estimated to be 70-80% and about 85-95% for liquid-based cytology tests.[2]

The most common cause of cervical carcinoma and dysplasia is HPV. The persistent high-risk type HPV infection of the cervical epithelium appears to trigger neoplastic progression.[3,4] p16INK4a (p16), has been relatively well established as a surrogate molecular marker of Human Papilloma Virus (HPV) related squamous dysplasia in cervical biopsy specimens with excellent inter- and intraobserver reproducibility. However, the main challenge in applying p16 to cytology preparations is the inherent difficulty in reproducible and objective interpretation of p16 immunoreactivity in whole cells with nucleus enveloped by surrounding cytoplasm due to the obscuring cytoplasmic immunoreactivit especially with relatively few diagnostic intact cells.[5,6,7]Performing immunohistochemistry (IHC) on cell block sections of cytologic specimens could handle this limitation.[8] . Present study was undertaken to investigate the feasibility and the role of cell block preparations in identifying significant neoplastic and preneoplastic lesions of the uterine cervix and to assess the feasibility of using p16INK4a immunohistochemical staining patterns on cell blocks to identify significant preneoplastic cervical lesions.

MATERIAL AND METHODS

The cell blocks were prepared from residual material from liquid based cytology of cervical brushings from women with positive cytopathological interpretation of squamous epithelial lesions (i.e ASCUS and above). P-16 immunoreactivity was evaluated in cell block sections in 50 cases (40 cases with positive cytopathology diagnosis + 10 cases which are negative for intraepithelial lesion or malignancy i.e. NILM) with adequate cellularity on a cell block.

Inclusion Criteria

1. Cases with positive cytopathological interpretation of squamous epithelial lesions.
2. Cases in which follow up cervical biopsy positive for dysplasia/HPV DNA testing reported positive.

Exclusion Criteria

1. Cases with cell blocks of inadequate cellularity.
2. Patient on chemo-therapeutic treatment currently or in past.

Only specimens that had 1 mL of residual fluid with visible, floating, tissuelike fragments after preparation of BDPrep slides were included. Cervicovaginal cytology specimens were collected in PreservCyt solution, and BDPrep slides were prepared, screened and interpreted using the 2014 Bethesda reporting system.

Cell-Block Preparation – Residual specimens collected in PreservCyt solution were used for thrombin cell block preparation for all patients. Cell blocks were prepared primarily according to the method described by Keyhani-Rofaga and Vesey-Shecket.43 The steps of the thrombin cell block preparation method are as follows:

1) The PreservCyt cell suspension remaining after performing BDPrep was centrifuged in 50 mL tubes for 5 minutes at 400g (1500 revolutions per minute);
2) Supernatant fluid was decanted or pipetted without disturbing the cell button;
3) The sediment was washed by resuspension in 5 mL normal saline followed by centrifugation for a second time;
4) 1 mL plasma (outdated human plasma) was added to resuspend the cell button, and cells were mixed thoroughly with the plasma;
5) Thrombin (topical, bovine origin; Dade Behring, Marbug, Germany) was diluted with 1 mL tap water, and half of the thrombin was used for each cell block;
6) Specimens were mixed gently by rotating the centrifuge tube and then were set aside until a clot formed (generally after 5 minutes);
7) A lens tissue (S/P; Allegiance Healthcare Corporation, McGaw Park, IL) was placed on top of a paper towel;
8) The clot was placed on the lens paper and squeezed gently with a small wooden applicator;
9) The concentrated clot was wrapped with lens tissue; and

10) The specimens were fixed in 10% neutral buffered formalin for 10 minutes.

The specimens then were transferred to a labeled tissue cassette and were submitted for histologic processing. Ten tissue sections, each measuring 4 m in thickness, were cut from the cell blocks, and were stained with hematoxylin and eosin for morphologic evaluation. Immunohistochemical Staining The number of diagnostic cells in the cell block material will be judged to be sufficient for immunohistop16INK4a and Ki-67 in Cervical Cell Blocks. Cell block sections were deparaffinized and hydrated. Antigen retrieval was performed using 1X target retrieval buffer (Dako, Carpinteria, CA), which was preheated until a thermometer placed in the steam chamber read at least 95 °C. Slides were dipped in hot water before being placed in 1X target retrieval working solution in a steamer (Black & Decker, Towson, MD) for 25 minutes. After steaming, slides were cooled to room temperature for 20 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide. Slides were then immunostained using the Envision system (Dako). The antibodies to p16INK4a (mouse monoclonal Ab-7, clone 16P07; NeoMarkers, Fremont, CA) were diluted 100-fold in antibody dilution buffer (Dako). Slides were incubated for 25 minutes and 20 minutes in the solutions for p16INK4a and 3,3-diaminobenzidine tetrahydrochloride was used as the chromogen. Paraffin blocks of adenosquamous carcinoma of lung were used as positive controls for p16INK4a.

Interpretation of Immunohistochemical Stains
Immunohistochemically stained slides were graded without any knowledge of the diagnosis rendered on the cell blocks or the ThinPrep slides. The following 4 parameters were evaluated45:

1) The percentage of positive cells, which was assessed semiquantitatively as < 10% or >_10% of cells having positive staining for p16INK4a (for p16INK4a, nuclear or nuclear and cytoplasmic staining was considered to be indicative of positivity;
2) The intensity of staining, which was graded subjectively as 0, +,++,+++ or and was grouped into 2 categories (weak [0,+ , ++or] and strong [+++]);
3) Full-thickness epithelial staining, which was defined by the presence of positively stained cells throughout all layers, from the base to the top of an epithelial fragment, in diagnostic strips; and
4) The number of positively stained cells in close contact, with close contact being defined by the presence of at least 2 adjacent positively stained squamous epithelial cells

Statistical Analysis
The chi square test was used for statistical analyses , and $p<0.05$ were considered indicative of statistical significance

RESULTS
Out of 50 samples, ASCUS (45%) was the most common epithelial cell abnormality followed by LSIL (40%). There were 72% concordant diagnoses on cell block and cytological analysis.18% cases showed lesser degree of epithelial cell abnormality on cell block while 10% cases showed higher degree of epithelial cell abnormality on cell blocks prepared. 6 specimens labeled as ASCUS on cytology were re classified as 3 NILM, 2 LSIL, 1 HSIL on cell block that were more beneficial with respect to patient follow-up and management. 6 specimens labeled as LSIL on cytology were re classified as 4 ASCUS and 2 HSIL on cell block analysis. 2 specimens labeled as HSIL on cytology were diagnosed as 1 ASCUS and 1 LSIL on cell block analysis.

Immunostaining of p16INK4a on cell blocks exhibited a statistically significant association ($p<0.05$)) with the presence of significant lesions on either cell block or cytological analysis. In current study, we found a) NILM cytology (10):1 (10%) sample was p16 positive. b) ASCUS cytology (18): 6 samples (33%) were p16 positive. C) LSIL cytology (16): 11 (69%) were p16 positive D) HSIL cytology (6): 6(100%) samples were p16 positive. Specimens reported as HSIL/SCC were found to have high intensity and diffuse staining for p-16 and have a greater percentage of positively stained cells compared with normal specimens and specimens exhibiting features of LSIL and ASCUS. Cytological diagnosis and p16 staining on cell blocks prepared were correlated with cervical biopsies wherever possible. 2/6 p-16 positive ASCUS samples showed strong intensity staining which were reported as 1 LSIL and 1 HSIL on cell block analysis. Also tissue follow up was present for these samples which were reported as CIN 1 and CIN 2 respectively. 2/11 P-16 positive LSIL samples which showed strong intensity staining, 2 of which were reported as HSIL on cell block analysis and reported as CIN 2 on tissue follow up. While 1/11 p-16 positive LSIL samples also showed strong positivity with cytological and cell block analysis reporting it as LSIL while on tissue follow up, it was reported as CIN 2

Table 1 Correlation Of Cell Block Diagnosis And Cytological Diagnosis

Cell block diagnosis	Cytological diagnosis				
	NILM	ASCUS	LSIL	HSIL/SCC	TOTAL
	No.	No.	No.	No.	No.
NILM	10	3	0	0	13
ASCUS	0	12	4	1	17
LSIL	0	2	10	1	13
HSIL/SCC	0	1	2	4	7
TOTAL	10	18	16	6	50

Table 2 Intensity Of P16 Staining On Cell Blocks According To Cytology Diagnosis

P16 STAINING INTENSITY ON CELL BLOCK	ACCORDING TO CYTOLOGICAL DIAGNOSIS			
	NILM	ASCUS	LSIL	HSIL
	No.	No.	No.	No.
Strong positivity	0	2	3	5
Weak positivity	1	04	8	1
Negative	9	12	5	0
Total	10	18	14	6

X^2: 27.136; p=0.001

Table 3 Intensity Of P16 Staining On Cell Blocks According To Cell Block Diagnosis

P16 STAINING ON CELL BLOCK	ACCORDING TO CELL BLOCK DIAGNOSIS			
	NILM.	ASCUS.	LSIL	HSIL
	No.	No.	No.	No.
Strong positivity	0	0	3	7
Weak positivity	1	6	7	0
Negative	12	13	3	0
Total	13	17	13	7

X^2: 47.699; p=0.001

Table 4 Correlation Of P16 Immunostaining On Cell Blocks Sections Of LBC With Biopsy

Cytology Diagnosis	P16 on cell block	Cervical biopsy diagnosis		
		CIN 1	CIN 2	CIN 3
		No.	No.	No.
ASCUS (2)	Positive (2)	1	1	0
LSIL (8)	Positive (8)	5	3	0
HSIL/SCC (6)	Positive (6)	1	2	3

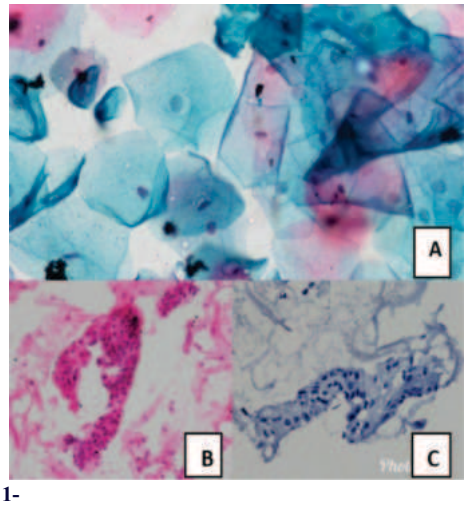

Image 1-
A) Shows features of ASCUS on cytology (PAP stain, x400)
B) Shows features of ASCUS on cell block (H&E stain, x100)
C) shows negative p-16 staining on cell block diagnosed as ASCUS (P-16 IHC, x100)

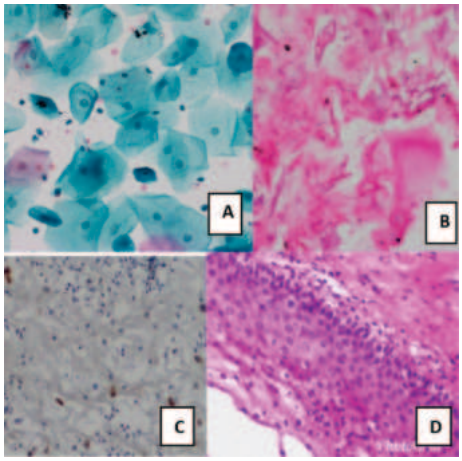

Image 2-
A) shows features of LSIL on cytology (PAP stain, x400)
B) shows features on HSIL on cell block (H&E stain, x100)
C) shows strong p-16 staining on cell blocks diagnosed as HSIL on cell block (p-16 IHC, X100)
D) shows follow up cervical biopsy showing features of CIN 2 (H&E stain, x400)

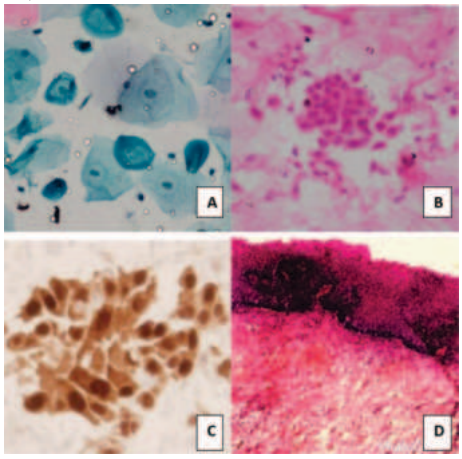

Image 3-
A) shows features of HSIL on cytology (PAP stain, x100)
B) Shows features of HSIL on corresponding cell block (H&E stain, x100)
C) Shows strong and diffuse p-16 staining for HSIL labeled specimens on cell block (p-16 IHC, x400
D) Shows features of CIN 3 on follow up cervical biopsy (H&E stain, x100)

DISCUSSION

Cell block preparations have been employed frequently in past for fine needle aspirations and non gynaecological cytology. It has lead to a better diagnostic approach to cases with malignancies as it has better architectural patterns and better cellularity which provides additional information.[9,10] Leung and Bedard found that all the cases with adequate material could be diagnosed on a cell block preparation.[11] The addition of cell block preparation from various exfoliations sources increases the sensitivity of detecting malignancies and also has the ability to reduce false-positive interpretations.[12] However, the cell block is a relatively new diagnostic technique for cervical lesions analysis. [13-22] For the purposes of the current study, cytologic analysis was considered to be the gold standard and was correlated with cervical biopsy wherever possible.

In current study, 72% of cell diagnosis agreed with cytological diagnosis. The observed level of concordance is similar to what has been reported by Akpolat et al[15], 73% Keyhani-Rofaga and Vesey-Shecket[13], 69%, Li Yu et al[23] , 75.4% in their study of cell block preparations from fluid based cervical samples.

It has been reported that the cell blocks are useful in (a) repair versus invasive squamouscell carcinoma, (b) immature squamous metaplasia versus HSIL, (c) atrophic changes versus HSIL, and (d) AGUS versus tubal metaplasia or inflammatory changes. Also Cell block results helped the ThinPrep Pap diagnosis in 20% of specimens and were critical to the diagnosis in 5% in the study conducted by k Richard et al.[24] One issue that remains controversial is the diagnosis of LSIL specimens on cell block. Some authors have concluded that cell blocks are useful in the diagnosis of LSIL,[13,15] whereas other authors do not agree.[24] Cell block preparations were not helpful in differentiating ASCUS from LSIL in 43 of 45 specimens analyzed by Richard et al.[24] Akpolat et al[15] also advocated the use of cell blocks in clarification of diagnosis of ASCUS. 11 specimens that were diagnosed as ASCUS on cytology were given specific diagnosis on cell block.

Similarly, in current study, 6 specimens labeled as ASCUS on cytology were re classified as 3 NILM, 2 LSIL, 1 HSIL that were more beneficial with respect to patient follow-up and management. It is also believed that in most specimens showing lesser degree of epithelial abnormality on cell blocks was attributed to the deficiency of diagnostic cells in residual material from which the cell blocks were prepared. Based on our experience, cell blocks are helpful in diagnosis of LSIL and in reclassification of diagnosis of ASCUS.

P16INK4a has been established as a surrogate marker for HPV associated dysplasia in various studies in past which used either cervical biopsies, cytology specimens.[6,25-29] However, cell blocks made from residual fluid based cervicovaginal material have been utilized in a limited number of studies for p16 immunoexpression. Data suggest that p16INK4a is useful in diagnostic problems of cervical lesions, such as differentiating between atrophy or squamous metaplastic cells from squamous epithelial lesions.[25,28,29] A comparison of immunocytochemical staining for p16INK4a in SurePath preparations with cytopathologic diagnoses was reported by Saqi et al.[27] In that study, p16INK4a expression was detected in: a) 18% of specimens with nonneoplastic/nondysplasic findings, b) 50% of specimens containing atypical glandular cells, c) 74% of specimens containing LSIL, d) 90% of specimens containing HSIL, and e) 100% of specimens containing SCC/adenocarcinoma.

And Akpolat et al conducted the first study in 2004 investigating p16ink4a in thinprep cell blocks.[15] In current study, we found a) NILM cytology (10):1 (10%) sample was p16 positive. b) ASCUS cytology (18): 6 samples (33%) were p16 positive. C) LSIL cytology (16): 11 (69%) were p16 positive D) HSIL cytology (6): 6(100%) samples were p16 positive. Our findings are comparable with the study conducted by Saqi et al[26] and Bibbo et al.[30] reported staining in 74% of LSIL and 96% of HSIL cases. However Shidham et al[16] reported the majority of ASCUS (14 out of 14, 100%) and LSIL (37 out of 50, 74%) cases were negative for p16 in cell blocks prepared from cytology specimens and Bao et al,[21] also reported positive rate of immunostaining for p16INK4A on cell blocks in NILM, ASCUS, LSIL, HSIL, SCC was 0% (0/2), 18.18% (2/11), 46.43% (13/28), 89.66% (26/29), 100% (9/9), respectively. The reason for this discrepancy could be the variability of the criteria for interpretation of p16 immunoreactivity. A recent meta-analysis of 61 studies published until 2007 on p16 immunoexpression which included 27 studies on cytologic specimens and 34 studies on cervical biopsies.[31] The analysis concluded that p16 immunostaining correlated with severity of cytological/histological abnormalities. However, the reproducibility was limited due to insufficiently standardized interpretation of the immunostaining. In the current study, specimens reported as HSIL/SCC were found to have high intensity and diffuse staining for p-16 and have a greater percentage of positively stained cells compared with normal specimens and specimens exhibiting features of LSIL and ASCUS. The levels of p16 expression were significantly higher in HSIL/SCC group when compared with LSIL and non neoplastic groups ($P<0.01$).

2/6 p-16 positive ASCUS samples showed strong intensity staining which were reported as 1 LSIL and 1 HSIL on cell block analysis. Also tissue follow up was present for these samples which were reported as CIN 1 and CIN 2 respectively which indicates that there is a correlation between weak or strong p16 expressions in ASCUS-categorized smears with the presence of SILs in follow-up biopsies.

2/11 P-16 positive LSIL samples which showed strong intensity staining, 2 of which were reported as HSIL on cell block analysis and reported as CIN 2 on tissue follow up. While 1/11 p-16 positive LSIL samples also showed strong positivity with cytological and cell block analysis reporting it as LSIL while on tissue follow up, it was reported as CIN 2. Since all the patients with LSIL do not progress to higher grade lesions. P16 staining can be useful for triaging these patients for

closer follow up and/or further evaluation.78 Shidham et al. [16] also concluded that p16 on cell block sections of cervical cytology specimens with positive squamous interpretation including ASCUS, LSIL, ASC-H, LSIL-H, and HSIL showed distinct patterns with excellent correlation with biopsy results.

This p16 assisted approach can result in better triaging, reduced non indicated invasive procedures, reduced morbidity and potential overtreatment.[16]

CONCLUSION

In conclusion, cytological smears when supplemented with cell block preparation and application of p-16INK4a on cell blocks can help in better differentiation of high grade, low grade and non neoplastic lesions in case of diagnosis ambiguity and can lead to better triaging of patients reducing patient recall along with decreased non indicated invasive procedures.

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