Original Article

p16 immunostaining as a predictor of anal and cervical dysplasia in women attending a sexually transmitted infection clinic

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Abstract

Background: Carcinogenesis caused by human papillomavirus (HPV) leads to over-expression of p16 protein. p16 may act as a marker of HPV integration with host genome and serve as a surrogate marker of HPV oncogenesis. **Materials and Methods:** A single center study of 75 women (35 HIV-positive and 40 HIV-negative women) was conducted. Anal and cervical specimens were obtained for cytology and p16 immunostaining. **Results:** The sensitivity of p16 to diagnose anal and cervical dysplasia was 50% and 58.8%, respectively, whereas specificity was 98.6% and 100%, respectively. Positive predictive value for anal and cervical was 75% and 100%, whereas negative predictive value was 95.8% and 89.2%, respectively. A strong relationship between the grade of dysplasia and intensity of p16 immunoscore was observed (Pearson correlation $r = 0.666$, $P < 0.0001$ and $r = 0.496$, $P < 0.0001$ for anal and cervical, respectively). **Conclusion:** p16 immunostaining with greater specificity for high-grade lesions may improve the diagnostic accuracy, especially for high-grade lesions which have a high risk of progression to malignancy and thereby necessitate treatment.

Key words: Anal intraepithelial neoplasia, cervical intraepithelial neoplasia, human papillomavirus, p16 immunohistochemistry, sexually transmitted infection clinic, women

INTRODUCTION

Human papillomavirus (HPV) is thought to be the principle etiologic agent responsible for the development of anogenital squamous cell carcinoma (SCC) and their precursors, squamous intraepithelial lesions (SILs).[1] Chronic HPV infection has been implicated to have a malignant potential and persistent infections of cervix and anal canal with oncogenic or high-risk types of HPV is an important factor for the development of cervical cancer and anal cancers, respectively.[2,3] High-risk HPV types (most commonly HPV 16) have been identified in more than 99% cases of cervical cancer.[2] Likewise, HPV has been detected in 78% of anal carcinoma; 90% in female patients and 63% in male patients.[3]

Carcinogenesis caused by HPV is characterized by increased levels of p16 protein immunoexpression.[4] It has been proposed that HPV-DNA integration into the host DNA is critical in carcinogenesis.[5] This integration results in an upregulation of the transcription of E6 and E7 viral oncogenes, and the primary activity of E7 is the binding of Rb protein in the hypophosphorylated or inactive state. Rb protein is in a negative feedback loop

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with p16 INK4 expression. Thus, inactivation of Rb by HPV proteins results in a paradoxical overexpression of p16 INK4a, normally a cell cycle inhibitor.[4] Therefore, p16 may act as a marker of HPV integration with host genome thereby serving as a surrogate marker of HPV oncogenesis.[6]

Cytology by using conventional Pap smears is a useful screening method for detecting anal and cervical SILs. Pap smear has sensitivity between 47% and 62% and a high specificity of 60–95%.[7] Cytology has also been collaborated with results by other methods, such as p16 immunohistochemical staining because of low specificity of Pap smears. p16, Ki-67, and BD ProEx™C have each been shown to be helpful adjuncts in the diagnosis and grading of HPV–associated cervical intraepithelial neoplasia (CIN), of which p16 was the most sensitive and specific individual stain.[8] The p16 protein serves as a surrogate marker for the oncogenic activities of HPV in cervical epithelia and is well established in CIN and invasive cancer by many studies.[9] It has also been used as adjunctive means to enhance the diagnostic accuracy of anal specimens with a sensitivity and specificity for diagnosing high-grade anal intraepithelial neoplasia (AIN) ranging from 72% to 100% and 71% to 100%, respectively.[10,11] There are only limited studies analyzing the role of p16 in cervical cytological specimens,[12-15] and even lesser studies available on anal cytological specimens.[16,17] Further, the role of p16 in AIN has been evaluated in men, particularly men who have sex with men[18] and data on women appears to be lacking. Therefore, this study was planned to study the role of p16 in CIN and AIN in women attending our sexually transmitted infection (STI) clinic.

MATERIALS AND METHODS

This cross-sectional study conducted from November 2011 to April 2013 included 75 women (40 consecutive HIV-positive and 35 high-risk HIV-negative sexually active women), aged between 18 and 45 years, attending the STI clinic of an urban hospital and consenting to be a part of the study. The patients presented with an STI or reported for screening from the anti-retroviral therapy clinic or as part of contact tracing. "High risk" was attributed when any of the following history was elicited: Reporting multiple sexual partners, sex with a person having multiple sexual partners, anal intercourse, any STIs. The Institutional Research Ethics Committee approved the study.

After obtaining written informed consent, each subject was asked to provide a detailed history on their gynecological health and the presence of an STI. Following the questionnaire and history, a detailed examination was performed, and evaluation of STI carried out. Corresponding cervical samples and anal cytology specimens were taken with a moistened cytobrush by a single clinician both for cytology and p16 immunostaining. Both anal and cervical smears were fixed and stained according to the papanicolaou protocol as well as p16 immunostaining procedure using p16 primary antibodies (G175-405, BioGenex Laboratories, California, USA).

The cytosmears were then reviewed independently by the pathologist who was blinded from the clinical details of the patients to avoid bias. The minimum criterion for adequate cellularity was taken as >1000 cells for anal smears and >7000 cells for cervical smears. The diagnosis for both anal and cervical smears was classified according to Bethesda classification 2001[19] as negative for intraepithelial lesions; atypical squamous cells of undetermined significance (ASCUS); low-grade SILs (LSIL); ASCUS cannot exclude high-grade squamous intraepithelial lesion (HSIL); HSIL.

Evaluation of p16 immunostaining

p16 positivity was taken as a brown reaction product staining the nucleus or cytoplasm or both. It was interpreted by the pathologist who was blinded from both the clinical and cytological findings. Scoring of percentage positive tumor cells was done as following; 0% staining as negative, 0–5% as 1þ, 5–25% as 2þ, and over 25% as 3þ. The intensity of immunostaining was taken as 1þ, 2þ, and 3þ depending on the positivity. Immunohistochemistry score then was obtained as a product of percentage positive tumor cells (0–3) and staining intensity score (0–3), thereby achieving a maximum score of 9.

Statistical evaluation

All statistical analysis was performed using SPSS Statistics software version 17.0 (SPSS Inc., Chicago, IL, USA). The threshold of significance was set at $P < 0.05$ (two-tailed) and $P < 0.01$ was taken as highly significant. The correlation of grade of both anal and cervical dysplasia with anal and cervical p16 intensity and immunoscore, respectively, was done by calculating Pearson's Chi-square coefficient.

RESULTS

Overall, 52% had an associated STI. The genital discharge was the most common STI, present in 34.6% of the patients. Genital warts were present

in six study subjects (10.2%). Patients without cervical dysplasia had significantly higher genital warts as compared to patients with cervical dysplasia ($P = 0.006$).

Abnormal cervical cytology was evident in 37.3% of the study subjects; LSIL was observed in 13 (17.3%) smears and HSIL in 4 (5.3%) smears. Abnormal anal cytology was present in 8% of the study subjects; LSIL was observed in 5 (6.7%) smears and HSIL in 1 (1.3) smears.

Anal p16 immunostaining

A total of 4 (5.3%) smears were positive for p16 and amongst them 2 (50%) were LSIL, 1 (25%) was HSIL and 1 (25%) was normal on anal cytological evaluation [Table 1]. All patients with ASCUS, reactive nuclear changes, and normal anal cytology were negative for p16 immunostaining. The nuclear staining pattern was observed in 1 (25%) and cytoplasmic staining pattern was seen in 3 (75%). Among cells that stained positive for p16, the mean p16 percentage positivity, mean p16 intensity score and the mean p16 immunoscore were 0.08 ± 0.36, 0.07 ± 0.30, and 0.11 ± 0.54, respectively. Among the six smears with dysplasia p16 positivity was seen in 3 (2 of 5 with LSIL and in the 1 HSIL) thereby, the sensitivity of p16 to diagnose anal dysplasia was 50%, whereas specificity was 98.6%. The positive predictive value (PPV) was 75%, whereas the negative predictive value (NPV) was 95.8%. Pearson's coefficient of correlation for p16 immunoscore and grade of dysplasia showed a positive correlation which was statistically highly significant ($r = 0.666$, $P = 0.00$) [Table 2].

Cervical p16 immunostaining

A total of 10 (13.3%) smears were positive for p16 and among them 60% were LSIL and 40% were HSIL. Of the total 13 patients who had LSIL on cervical cytology 6 (46.2%) were positive for p16 staining and 100% p16 immunostain positivity was observed in all the 4 patients with HSIL on cervical cytology [Table 1]. All patients with ASCUS, reactive nuclear changes, and normal anal cytology were negative for p16 immunostaining. The nuclear staining pattern was observed in 20% and cytoplasmic staining pattern was seen in 80%. Among the patients positive for p16 staining, the mean p16 percentage positivity, mean p16 intensity score mean and p16 immunoscore was 0.23 ± 0.64, 0.23 ± 0.64, and 0.44 ± 1.5, respectively [Figures 1-3]. The sensitivity of p16 to diagnose cervical dysplasia was 58.8%, whereas specificity was 100%. The PPV was 100% whereas the NPV was 89.2%. Pearson's coefficient of correlation for p16 immunoscore and grade of dysplasia showed a positive correlation which was statistically highly significant ($r = 0.496$, $P = 0.00$) [Table 2].

Table 1: Patients with anal and cervical cytology positive for p16

Patient number	Serostatus	Cytology	p16 percentage positive score	p16 intensity score	p16 immuno-score
Anal specimens					
1	Positive	Normal	1	1	1
2	Positive	HSIL	2	2	4
3	Negative	LSIL	2	1	2
4	Negative	LSIL	1	1	1
Cervical specimens					
1	Positive	LSIL	3	3	9
2	Positive	LSIL	3	3	9
3	Positive	LSIL	2	1	2
4	Positive	LSIL	1	1	1
5	Positive	HSIL	1	2	2
6	Positive	HSIL	2	2	4
7	Negative	HSIL	2	1	2
8	Negative	LSIL	1	1	1
9	Negative	HSIL	1	2	2
10	Negative	LSIL	1	1	1

LSIL=Low-grade squamous intraepithelial neoplasia; HSIL=High-grade squamous intraepithelial neoplasia

Table 2: Correlation of p16 with grade of anal and cervical dysplasia

Grade of dysplasia	p16 immunostaining score**		
	Pearson correlation	Significant (two-tailed)	*n*
Grade of anal dysplasia	0.666***	0.000	75
Grade of cervical dysplasia	0.496***	0.000	75

**Calculated as multiplication product of p16 intensity score and p16 percentage positivity of cells; ***Correlation is significant at the 0.01 level (two-tailed)

DISCUSSION

HPV is an epitheliotrophic double stranded DNA virus which causes cellular and oncogenic changes. At molecular level p16INK4a and P53 are tumor suppressor genes and key targets in the loss of cell cycle control. The HPV oncoproteins, E6 and E7 increase degradation of p53 and interfere with pRb function leading to upregulation of p16 by the loss of negative feedback control.[4] Overexpression of p16 in HPV infection has been demonstrated in a high percentage of cancers[20] and it has been suggested that it may serve as a surrogate biomarker of oncogenic HPV infection in predicting HPV-related tumors.[6,20] The gene encoding p16, also an inhibitor of human cyclin-dependent kinase 4, has recently been mapped to 9p2l, the site for the multiple

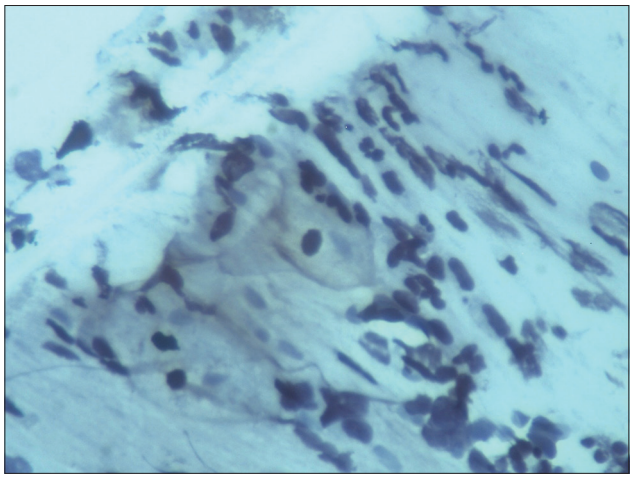

Figure 1: p16 immunostaining (×40) showing faint p16 staining in cervical squamous cells

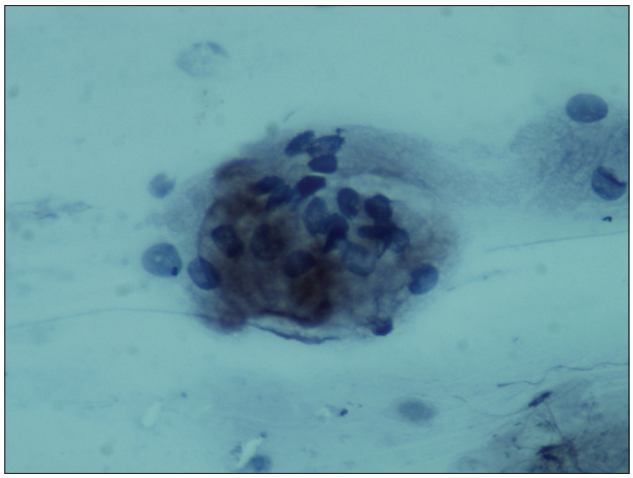

Figure 2: p16 immunostaining (×40) showing moderate p16 staining in cervical squamous cells

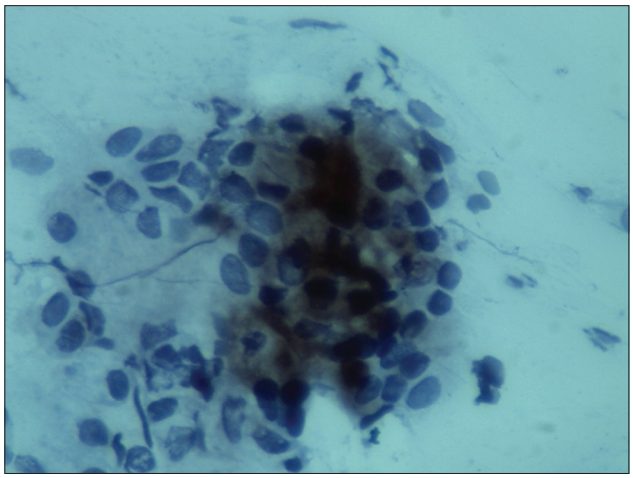

Figure 3: p16 immunostaining (×40) showing strong p16 staining in a group of cervical

tumor suppressor locus.[21] p16 is a cyclin-dependent kinase inhibitor that negatively regulates cell proliferation by inhibiting hyper-phosphorylation of pRb via the Cdk4/6. Overexpression of p16 protein is a consequence of pRb targeted inactivation from HR-HPV E7 protein.[4,20] Its role in cervical cancer has been well evaluated as a diagnostic tool as well as a marker of disease progression.[22] Hence, its use for evaluation of anal dysplastic lesions has been proposed due to the similarity in natural history and progression of the disease in anal canal and cervix.[17]

Pap smear evaluation has some limitations in diagnostic utility. Even though it has a high sensitivity, the specificity remains low.[7] Furthermore, it has a high rate of atypical cells of undetermined significance leading to misdiagnosis. The presence of atypical keratinized squamous cells creates a diagnostic dilemma as these can vary from benign to markedly atypical, thus leading to false diagnosis of malignancy. Moreover, reactive nuclear changes in the presence of infections may further lead to misdiagnosis because of their association with SCC.[17] Further, an inter-observer variation has been noticed in the interpretation of cervical and anal specimens for CIN and AIN, respectively. Therefore, this labor intensive complex method of screening and diagnosis and the human judgment based outcome propagates a need to optimize the interobserver reproducibility of cytological as well as histological interpretations.[23] Hence, p16 immunostaining may provide the advantage of being an effective tool to screen anal dysplastic lesions as well as improve the inter-observer agreement for the diagnosis of SILs.[24]

Moreover, p16 expression has been studied to show a correlation with progression of the lesion.[25] A strong p16 overexpression has been demonstrated to precede detection of integration of high-risk HPV, which may also play an important role in early detection of high-risk lesions.[22] Furthermore, p16 expression has been shown to decrease spontaneously with the regression of the lesion.[25] Therefore, it may be a useful adjunct for evaluation of treatment response in HPV infections.

However, the literature on the use of p16 on cervical and anal specimens is still limited. The role of p16 CIN and AIN have been evaluated mostly in biopsy specimens and studies on cytological smears are limited, with few studies on cervical cytological smears[12-15] and still fewer on anal cytological smears.[16,17] The role of p16 in AIN have been evaluated mainly on men and data on women appears to be lacking.[18]

In this study, 50% sensitivity and 98.6% specificity for anal smears were obtained. This is in concordance

Table 3: Studies analyzing the role of p16 in anal cytology

Author (year, place)	Study population	Positive stain, % (*n*)	Grade of dysplasia, % (*n*)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Tramujas da Costa E Silva *et al.*[16] (2011, Brazil)	190 HIV-positive patients (both men and women)	-	-	31	81	-	-
Darvishian *et al.*[17] (2006, USA)	43 cytology smears, 29 HIV-positive patients (17-males, 12-females)	65 (28)	LSIL-6.9 (3) HSIL-51 (22)	72	71	93	33
Arora *et al.*[18] (2014, India)	65 MSM cytology (31 HIV -positive and 34 HIV-negative)	20 (13)	LSIL- 64.3 (9) HSIL-100 (4) Normal-0	72.3	100	100	92.3
Present study (2015, India)	75 women cytology (40 HIV-positive and 35 high risk HIV-negative)	5.3 (4)	LSIL-40 (2) HSIL-100 (1) Normal-1.4 (1)	50	98.6	75	95.8

AIN=Anal intraepithelial neoplasia; ASCUS=Atypical squamous cells of undetermined significance; ASCUS-H=Atypical squamous cells of undetermined significance cannot exclude high-grade squamous intraepithelial lesion; HIV=Human immunodeficiency virus; PPV=Positive predictive value; NPV=Negative predictive value; LSIL=Low-grade squamous intraepithelial neoplasia; HSIL=High-grade intraepithelial neoplasia; MSM=Men who have sex with men

with findings of the few studies on p16 for anal smear, which have demonstrated sensitivity ranging from 31% to 72% [Table 3].[16,17] Moreover, p16 showed 40% positivity for LSIL lesions and 100% positivity for HSIL lesions. All except 1 patient with ASCUS, reactive nuclear changes, and normal anal cytology were negative for p16 immunostaining. This reflects on the high specificity of p16 immunostaining. Further, a strong relationship between the grade of anal dysplasia and intensity of p16 immunoscore (Pearson correlation $r = 0.666$, $P < 0.0001$) on anal smears was also observed. This correlation has not been attempted in most of the previous studies.[16,17] This substantiates the importance of p16 in the diagnosis of SIL lesions which have a higher risk of progression to malignancy.

We obtained a sensitivity and specificity for p16 in cervical cytology of 58.8% and 100%, respectively. A sensitivity ranging from 75%[26] to 95%[14] and specificity ranging from 23.3%[14] to 92%[27] has been previously reported. Moreover, p16 showed 46.2% positivity for LSIL lesions and 100% positivity for HSIL lesions. All patients with ASCUS, reactive nuclear changes, and normal anal cytology were negative for p16 immunostaining. This reflects on the high specificity of p16 immunostaining. A direct relationship between grade of cervical dysplasia and intensity of p16 staining has also been reported.[22] This study also demonstrated a strong relationship between grade of cervical dysplasia and intensity of p16 immunoscore (Pearson correlation $r = 0.496$, $P < 0.0001$) for cervical smears. This strong intensity staining has been found to be associated with integrated high-risk HPV positivity, whereas episomal or absence of high-risk HPV has shown to show a lower intensity or negative staining.[22]

These findings highlight the importance of p16 in the diagnosis of high-grade SIL lesions, having a high risk of progression to malignancy and necessitating treatment.[28] However, it is to be noted that p16 is not a perfect surrogate marker for HPV as confounding factors such as genetic alterations in pRb/Cdk pathway, and technical factors may influence diagnosis.[22] Furthermore, further evaluation is needed to prove its role for evaluation of treatment response in HPV infections. Apart from p16, Ki-67 has also been evaluated as a useful adjunct to improve the pathologic diagnosis of cervical and anal biopsies. These can form an important component in the evaluation of cytosmears that are difficult to assess for CIN and AIN by morphology alone and also identify patients that are at higher risk for progression to malignancy and therefore need intensified screening.

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Conflicts of interest

There are no conflicts of interest.

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