Evaluation of P16^{INK4A} as a Biomarker in Cervical Intraepithelial Neoplasia

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ABSTRACT

Introduction: Cervical screening would benefit from a test based on a disease-specific biomarker that identifies high grade lesions, which could also indicate the presence of early precancerous lesions that have a high risk of progression to cancer. One such potential biomarker is p16^{INK4A}. Objective of the study was to study the biomarker p16^{INK4A} expression by immunostaining in cervical intraepithelial neoplasia.

Material and method: Experimental study conducted from November 2009 to April 2011. 1500 women were screened for cancer cervix using conventional Pap test, VIA and VILI. Women having positive results underwent colposcopy and biopsy if required. P16^{INK4A} expression in biopsy samples was studied using immunohistochemistry.

Results: All test positive cases n= 235, underwent colposcopy. Colposcopic abnormalities were detected in n=83 and biopsy proven cervical intraepithelial neoplasia(CIN) in n=15. P16^{INK4A} expression was seen in eight of 15 CIN cases. The strength of positivity of P16^{INK4A} was higher in CIN 2 and CIN 3 cases as compared to CIN 1. Also, the pattern of staining differed among various grades of CIN.

Conclusion: P16^{INK4A} expression was seen in majority of CIN 2 and CIN 3 lesions suggesting a higher grade lesion as compared to CIN 1. The discrimination between the lesions can be made without having to depend on traditional histopathology which gives a static picture of pre-invasive lesions of the cervix. This can help in differentiating high grade lesions from the lower grades, thereby helping in appropriate management of the cases.

Keywords: Cervical intraepithelial neoplasia, P16INK4A, PAP smear, colposcopy, cervical cancer

INTRODUCTION

Cervical carcinoma is a leading cause of mortality and morbidity among women especially in the developing countries.[1] Current cervical cancer screening tests include Papanicolaou (Pap) test; visual inspection after the application of acetic acid (VIA); visual inspection after the application of Lugol's iodine (VILI) and Human Papillomavirus (HPV)-DNA testing.

The Pap test has decreased cervical cancer incidence substantially in the countries with regular screening programs.[2] However, the suboptimal reproducibility of the Pap test has to be compensated by frequent retesting.

In developing countries simple, inexpensive visual based screening programs can be applied to a large population.[3,4] VIA is a valuable screening tool in low-resource settings; however it has low sensitivity and specificity.[5]

Randomised controlled trials published recently have demonstrated that HPV testing can be efficiently integrated into primary screening, either as an adjunct to cytology or as a sole primary test.[6,7] A single HPV DNA test although confirms infection by the virus, it does not discriminate between transient and persistent infection.[8] The discrimination between the two types of infection is crucial as persistent infections can progress to cervical neoplasia.[9]

Thus cervical screening would benefit from a test based on a disease-specific biomarker that identifies high grade lesions. Such a marker would be useful if it could also indicate the presence of early precancerous lesions that have a high risk of progression to cancer. One such potential biomarker is p16^{INK4A}. Some preliminary studies suggest that p16^{INK4A} positive low grade lesions showed a greater frequency of progression than p16^{INK4A} negative lesions.[10]

p16^{INK4A} is a gene that is expressed by host cells in response to infection, and is not normally expressed in non-transformed cells.[11] In cervical carcinomas, the viral DNA of hrHPV (i.e. HPV 16 and 18) is integrated into the host genome at the E$_2$ region, resulting in overexpression of the oncoproteins E6 and E7. The E6 binds with the host p53 tumor suppressor gene protein product and degrades it, thereby disrupting its cell regulatory role.[10] The E7 binds to and inactivates the tumor suppressor retinoblastoma protein (pRB) that inhibits the progression of cells into the S-phase. Consequently, loss of pRB function should result in the release of the p16^{INK4A} gene from negative transcriptional feedback control in the

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respective cells and in marked overexpression of p16^{INK4A} gene product.

Several properties of p16^{INK4A} make this protein a promising biomarker for HPV-related cancers; expression is directly linked to the HPV oncogene action, since continuous expression of E7 is necessary to maintain a malignant phenotype in HPV-associated cancer.[12] The expression of p16^{INK4A} seems to be independent of the HPV type causing the oncogenic infection, obviating the need to detect different HPV types in DNA and RNA assays.

MATERIAL AND METHODS

This is an experimental study conducted at the University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, India from November 2009 to April 2011.

1500 women were screened for cancer cervix using conventional Pap test, VIA and VILI. Women who had positive results in any of the above tests underwent colposcopy. Biopsy was taken from the abnormal areas detected at colposcopy. P16^{INK4A} expression in the biopsy samples was studied using immunohistochemistry. A written informed consent was taken from all patients. Sexually active women in the age group of 20-50 years were included in the study. Women with an obvious cervical growth, acute cervicitis, prior surgery on the cervix, postmenopausal and pregnant women were excluded from the study.

A detailed history including marital, sexual, obstetric, menstrual and personal history was taken. This was followed by a detailed examination which included general physical and systemic examination. Prior to per vaginal examination following screening tests were performed.

1. Screening tests
    Papanicolaou test (PAP test): Conventional PAP test was performed and results were reported according to the Bethesda 2001 System of reporting cervico-vaginal smears. A Pap test of ASCUS and above was taken as positive
    B. VIA and VILI: 5% acetic acid was applied liberally using a cotton swab soaked in acetic acid over the exposed cervix. The findings were read after one minute of application. After carefully recording the findings, Lugols's iodine was applied with a cotton swab on the cervix. The cervix was examined for any iodine non-uptake areas. The outcome of VIA and VILI was interpreted as per the International Agency for Research on Cancer (IARC) guidelines.[13]

2. Colposcopy - All women having positive result through either PAP test, VIA or VILI underwent colposcopy. In dorsal position, cervix was exposed and using the colposcope, the transformation zone of the cervix was visualized for any abnormal areas. The abnormal areas included acetowhitening, atypical vessels, punctations, mosaic pattern, iodine negativity. A biopsy was then taken from these abnormal areas.

3. Histopathology - A colposcopy directed single/ multiple punch biopsy of the suspicious areas was taken and transported to the pathology lab in 10% formalin. The results of the biopsy were reported as a) Chronic cervicitis a) CIN 1 b) CIN 2 c) CIN 3 d) Microinvasive cancer e) Invasive cancer.
    Immunostaining for p16^{INK4A} - For immunohistochemistry, representative sections from paraffin block of cervical biopsy were taken on poly-L-lysinate coated slides. Antigen was retrieved by microwave heat method using citric acid buffer at pH 6.0. Immunohistochemistry was performed using standard technique by Streptavidin biotin system using DAB as chromogen. Patterns of p16^{INK4A} were categorised as positive or negative. Positive p16^{INK4A} was defined as presence of nuclear staining or diffuse cytoplasmic staining. Negative p16^{INK4A} was defined as absence of any staining or presence of focal cytoplasmic staining. Specifically, diffuse staining was defined as a continuous staining of cells in the basal and parabasal layers (with or without staining of superficial squamous cell layers). Focal staining was defined as non-continuous staining of isolated cells or small cell clusters, usually not located in the basal and parabasal layers. Degree of nuclear p16^{INK4A} expression in positive cases were expressed as percentage of positive cells. Strength of positivity was compared to a positive control which could be run with each batch of immunostaining. The strength of reaction was graded as 1+, 2+ and 3+.

Positive control: Squamous cell carcinoma of esophagus
Negative control: Obtained by omitting the application of primary antibodies during the immunostaining process and using Tris Buffer Saline instead.

RESULTS

One out of the six samples of CIN-1 was damaged during the staining process. P16^{INK4A} was negative in four of the five CIN-1 biopsy samples that were stained. Only one CIN-1 biopsy showed positive P16^{INK4A}; with no cytoplasmic staining, 9% nuclear staining and strength of positivity 1+ (Figure 1).

P16^{INK4A} was positive in four of the five CIN-2 biopsy samples that were stained, with diffuse cytoplasmic staining. Nuclear staining in these cases ranged from 16% to 27% and strength of positivity was 2+ in three cases and 1+ to 2+ in one case. One CIN-2 biopsy sample showed negative p16^{INK4A} staining, with only focal cytoplasmic staining and negative nuclear staining. Positivity of p16^{INK4A} in CIN-2 was 80% (Figure 2).

P16^{INK4A} was positive in two of the three CIN-3 biopsy samples that were stained, with diffuse cytoplasmic staining. Nuclear staining in these two cases was 32% and 64% and strength of positivity was 2+ and 3+ respectively. One CIN-3 biopsy sample showed negative p16^{INK4A} stain (Figure 3).

P16^{INK4A} was positive in the one case of squamous cell carci-

noma that was detected in this study, with diffuse cytoplasmic staining, 90% nuclear positivity and strength of positivity 3+ (Figures 4). P16[INK4A] expression in the various cases of CIN/SCC has been shown in Table 1. P16[INK4A] immunostaining was also performed in 10 histopathologically proven cases of chronic cervicitis. P16[INK4A] was negative in eight of the 10 cases (Figure 5). In the two cases of chronic cervicitis in which P16[INK4A] was positive there was diffuse cytoplasmic staining and no nuclear staining.

DISCUSSION

A serious disadvantage of the grading by conventional histopathology is that the three distinct grades used in CIN can easily give a faulty static impression of a solidified sculpture, as if CIN were a static event, whereas in reality a CIN lesion is a dynamic process that can progress and persist but also regress. Compounding the above are the well-known issues of intraobserver and interobserver reproducibility, which, for grading of CIN, is far from perfect.[14] It is also difficult to distinguish CIN reliably from non-neoplastic lesions, resulting in either overtreatment or undertreatment. These points emphasise the need for adjuvant methods to interpret the actual morphological impression of a CIN lesion in dynamic terms rather than in static morphological grades. Without doubt, p16[INK4A] is the most widely available, robust, stable and strong predictive biomarker currently available for prognosticating CIN lesions. p16[INK4A] overexpression has been demonstrated in the vast majority of cervical precancers and cancers while in normal tissue, p16[INK4A] expression is found only rarely.[15] This was also shown in our study where among the 10 morphologically proven cases of chronic cervicitis p16[INK4A] only two showed diffuse cytoplasmic staining and no nuclear staining. Until now, despite several proposed evaluation strategies of p16[INK4A] in both cytology and histology, there is no general consensus for establishing threshold values above which a sample becomes "p16[INK4A]-positive."[16]
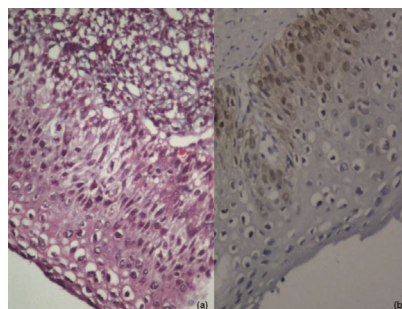

Figure-1: (a) H&E X 200: CIN I, (b) p16[INK4A] Immunoreactivity seen in lower 1/3 epithelium with some nuclei showing positivity in middle 1/3
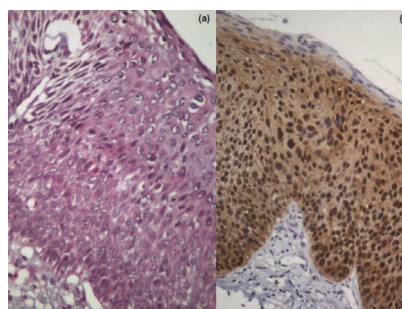

Figure- 2: (a) H&E X 200: CIN II, (b) p16[INK4A] Immunoreactivity: Nuclear positivity present throughout the epithelium with nuclei showing intense positivity in focal areas
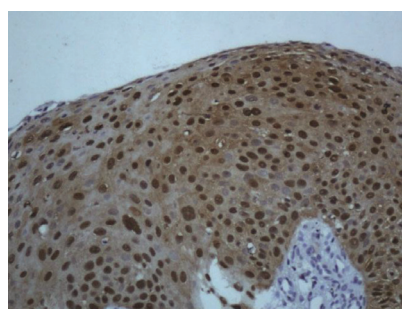

Figure-3: CIN III: p16[INK4A] Immunoreactivity seen in nuclei and cytoplasm

| S. No	Histopathology*	P16[INK4A], IHC**, result	P16[INK4A] cytoplasmic staining	P16[INK4A] nuclear staining	Strength of positivity
1.	CIN-1	Negative	Negative	Negative	Negative
2.	CIN-1	Negative	Negative	Negative	Negative
3.	CIN-1	Negative	Negative	Negative	Negative
4.	CIN-1	Negative	Negative	Negative	Negative
5.	CIN-1	Positive	Negative	9%	1+
6.	CIN-2	Positive	Diffuse	22%	2+
7.	CIN-2	Positive	Diffuse	18%	1+ to 2+
8.	CIN-2	Positive	Diffuse	16%	2+
9.	CIN-2	Positive	Diffuse	27%	2+
10.	CIN-2	Negative	Focal	Negative	Negative
11.	CIN-3	Negative	Negative	Negative	Negative
12.	CIN-3	Positive	Diffuse	32%	2+
13.	CIN-3	Positive	Diffuse	64%	3+
14.	Squamous cell carcinoma	Positive	Diffuse	90%	3+
*1 sample of CIN 1 lost during processing, **IHC: Immunohistochemistry					
Table-1: Cytoplasmic and nuclear p16[INK4A] expression in CIN and squamous cell carcinoma cases					
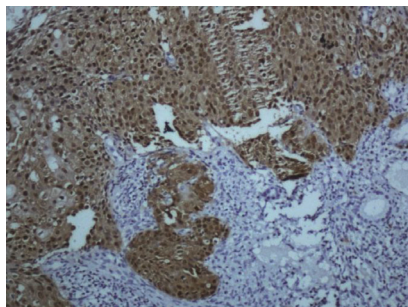
Figure-4: Squamous cell carcinoma. p16[INK4A] Immunohistoto-chemistry showing intense nuclear and cytoplasmic positivity

Figure-5: IHC X20 negative P16[INK4A] in chronic cervicitis

Klaes and colleagues[15], proposed a system which scored the distribution of p16 positivity on a semiquantitative scale as follows: negative (<1% of the cells were positive), sporadic (isolated cells were positive, but <5%), focal (small cell clusters, but <25% of the cells were positive), diffuse (>25% of cells stained positive).

In the present study patterns of p16[INK4A] staining defined was a modification of that of Klaes et al[15], where we reported cases as negative, focal or diffuse, and also graded nuclear positivity as a percentage.

CONCLUSION

Though p16[INK4A] has been analyzed in a number of studies, there is undoubtedly some way to go before we can say how they will "best fit" to improve the diagnosis of cervical neoplasia, as either stand alone or as adjunctive tests, in triage or in primary screening contexts. For this we need more clinical data, particularly sufficiently planned, longitudinal studies where the candidates are assessed alongside concurrent pathology.

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