Role of p16/Ki-67 Dual Immunostaining in Detection of Cervical Cancer Precursors

Diya Das, Moumita Sengupta, Keya Basu, Mona Tirkey, Chhanda Datta, Uttara Chatterjee
Department of Pathology, Institute of Postgraduate Medical Education and Research, Kolkata, West Bengal, India

Abstract

Background: Pap-smears-based cytology and human papilloma virus testing have their own limitations in detecting cervical precancerous lesions, and still need further standardization. Co-expression of p16\(^\text{ink4a}\) and Ki-67 can be used as additional biomarker. Aims: To study the role of liquid-based cytology and the dual immunostaining for p16/Ki-67 in predicting the presence of significant lesion in cases of mild cytological atypia. Materials and Methods: A prospective, cross-sectional study was performed in the Department of Pathology, in collaboration with Department of Obstetrics and Gynecology over 15 months including 545 patients. Immunocytochemistry followed by colposcopy-guided biopsy were performed in 52 cases with epithelial abnormalities. Results: Thirty-five cases (67%) were dual-stain positive among the cases with epithelial abnormalities. In the ASC-US and LSIL group, the sensitivity and specificity of the immunostaining in diagnosing CIN2+ lesions were 100 and 70% and 87.5 and 100%, respectively. p16/Ki-67 positivity also increased with cytological severity which in turn corresponded with histological findings: it reached from 33% in ASC-US to 100% in both HSIL and SCC categories. Conclusion: This dual immunostaining may potentially be a useful tool in the triage of the ASC-US and the LSIL group, considering the high sensitivity and specificity values.

Keywords: ASCUS, colposcopy guided biopsy, liquid based cytology, p16/Ki-67 immunostaining, LSIL

Introduction

Despite having a national cancer program since 1975, cervical cancer continues to be a major public health problem in India and in other developing countries, where it still leads the cancer-related cause of death. While there has been a significant improvement in the detection of precancerous and cancerous lesions of the cervix with Pap-smear programs, it is still not a full-proof standardized method. For example, different studies have shown that cytology has a sensitivity varying from 47 to 62% and specificity between 60 and 95% for the detection of high-grade cervical intraepithelial neoplasia (CIN2/CIN3). An estimated 5–20% of the tests also end up giving false-negative results in the general population. Approximately 30% of newly diagnosed cervical cancer patients have at least one previously false-negative Pap test result.

Recent years has seen a worldwide shift in the approach of cervical cancer screening programs from cytological evaluation to human papilloma virus (HPV) DNA testing by molecular assay. Although incorporated in the standard screening protocols in most setups, even this test has its limitations. In spite of a high sensitivity and NPV compared to the Pap-smear test, its specificity in detecting precancerous lesions of the cervix remains low, which is especially true for women under the age of 30 years. In view of the self-limiting nature of HPV infection in most of the cases, and the relatively low incidence of a productive viral infection phase converting into a transforming infection by virtue of viral oncogenic expression, it becomes imperative, especially in resource-limited countries, to come up with newer diagnostic modalities to diagnose transforming HPV infections of the cervix, to streamline the screening protocols.

A number of potentially useful biomarkers are being investigated for this purpose, and these include p16\(^\text{ink4a}\), HPV E6/E7 mRNA, and novel methylation assays.

Address for correspondence: Dr. Moumita Sengupta, Department of Pathology, Institute of Post Graduate Medical Education and Research, Kolkata - 700 020, West Bengal, India. E-mail: moumitasengupta83@gmail.com

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$p16^{INK4A}$ is the host cellular correlate of E7, the viral oncprotein. The Rb pathway deregulation is brought about by the overexpression of E7, which, in turn, activates a negative feedback loop and leads to a compensatory overexpression and cellular accumulation of $p16^{INK4A}$. Regarded as a reliable marker of precancerous lesion of the cervix, $p16$ can be detected in histology and cytology by immunostaining. However, interpretation of $p16$ staining requires additional morphological evaluation also, as it is possible for some benign cervical epithelial cells to show $p16$ expression too.

In this study, we have evaluated the role of liquid-based cytology (LBC) and a recently developed dual immunostain for $p16$ and Ki-67 in allowing identification of abnormal cells in cytology smears, and their role in predicting the presence of significant clinical lesion in cases of doubtful or mild cytological atypsia.

**Materials and Methods**

**Study population**

It was a prospective, population-based, cross-sectional study performed in the Department of Pathology, in collaboration with Department of Obstetrics and Gynecology, in a tertiary care institute in India, spread over a period of 15 months (January 2014 to March 2015).

Patients aged 18 years or above, presenting to the Gynecology Outpatient Department with symptoms of intermenstrual bleeding, postcoital bleeding, contact bleeding, excitation pain, excessive or offensive smelling vaginal discharge, dyspareunia, and pelvic pain syndrome were included in the study. Exclusion criteria were cases of previously diagnosed cervical cancer, pregnancy, prior therapy for cervical neoplasia (e.g., loop electrosurgical excision procedure (LEEP), LASER therapy, cold-knife conization, cryotherapy, and hysterectomy), and inability to give informed consent. An approval from the Institutional Ethics Committee was obtained for the said study.

A total of 1500 women attending the Gynecology Outpatient Department were initially approached for the study with conventional Pap-smear. Among them, 545 patients met the inclusion criteria and gave consent to participate in the study. These 545 cases were subjected to LBC. Of them, 53 cases showing abnormal cytological findings were subjected to immunocytochemistry. Eventually, 52 of these cases also underwent colposcopy-guided biopsy and histological examination, and one case was lost to follow-up.

**Manual liquid-based cytology**

Cervical cytology samples were collected using a Wallach broom device and transferred to SurePath® Preservative Solution (TriPath Imaging Inc., Burlington, NC 27215, USA). Each sample was processed manually as per instruction of manual method product insert provided by the manufacturer. The cytology slides were evaluated by a cytotechnologist and final diagnosis in each case was made by a trained cytopathologist according to the guidelines laid down by the Bethesda System of Reporting Cervical Cytology (2014). Slides that did not meet the squamous cellularity criteria as specified in the Bethesda reporting system were excluded from evaluation.

**Immunocytochemistry**

Another set of cytology slides were prepared from cells harvested from the homogenous cell mixture preserved in the initial phase of LBC preparation for each patients with abnormal cytological findings. $p16$/Ki-67 immunostaining of these slides was performed using the CINtec® Plus Kit (Roche mtm laboratories AG, Heidelberg, Germany) according to the manufacturer’s instructions. The primary antibody cocktail included a mouse monoclonal antibody (clone E6H4) against human $p16^{INK4A}$ (p16) protein and a rabbit monoclonal antibody (clone 274-11 AC3) directed at human Ki-67 protein. Positive staining yielded brown and red staining for p16 and Ki-67, respectively. Alcohol-free hematoxylin was used for counterstaining.

Following a preliminary screening done by a cytotechnologist for the presence of cells staining positively with both markers, the slides were reviewed and analyzed by two cytopathologists independently. A case was considered positive if one or more cervical epithelial cells showed simultaneous brown cytoplasmic stain (p16) and a red nuclear (Ki-67) irrespective of morphologic abnormalities. Slides without any cells showing dual immunoreactivity were considered negative.

**Colposcopy and histology**

Patients with abnormal cytological findings were referred for colposcopy. Accordingly, patients underwent colposcopy-guided biopsy at the gynecology clinic. Two pathologists with extensive experience in female genital tract pathology independently reported the tissue samples obtained by biopsy.

**Statistical analysis**

Data analysis was done with the help of Graph Pad Instat, Version 3 (Graph Pad Software, San Diego, CA, USA) and Prism Graph Pad, Version 5. Chi-square test for independence was done for evaluating association of $p16$/Ki-67 staining with cervical epithelial abnormality. All the categorical data were compared by Fisher’s exact test. A $P$ value $<0.05$ was taken as statistically significant.

**Results**

The study included 545 women with mean age of 36.8 years. Out of 545 patients who met the inclusion criteria, 9 had cytology smears unsatisfactory for evaluation. Of the rest 536 patients, 483 (90.1%) were diagnosed as noninvasive load monitoring, 15 (2.8%) had ASC-US, 5 (0.9%) had ASC-H, 12 (2.2%) had LSIL, 11 (2.0%) had HSIL, and 10 (1.9%) had invasive squamous cell carcinoma. Out of the 53 cases
of epithelial abnormality, 1 was lost to follow-up and 52 underwent colposcopy-guided cervical biopsy and histological examination in the same institute.

Table 1 shows the comparison of conventional Pap-stain and LBC in diagnosing cytological abnormalities. Table 1 also incorporates number of dual-stain positivity cases and their corresponding histological diagnoses.

Both LBC and conventional Pap-smear were done in each of the 545 patients. Considering histology as the gold standard, Table 2 shows that both sensitivity and specificity in detecting cervical epithelial lesions were more in LBC (97.14 and 76.47%, respectively) compared to conventional Pap cytology (87.18 and 69.23%, respectively). The positive predictive value (PPV) was the same for

### Table 1: Distribution of cases on conventional cytology and LBC along with their dual immunostain status and histological diagnoses

| Conventional cytology | Statistics | NILM | ASC-US | LSIL | ASC-H | HSIL | SCC | Total |
|-----------------------|------------|------|--------|------|--------|------|-----|-------|
| **NILM**              | Number     | 483  | 3      | 4    | 2      | 4    | 0   | 496   |
| p16/Ki67              | 1 (33%)    | 2 (50%) | 1 (50%) | 3 (75%) |
| CIN1                  | 1 (33%)    | 0     | 2     | 0    |        |      |
| CIN 2                 | 1 (33%)    | 0     | 1     | 0    | 1      |
| CIN3                  | 0          | 0     | 0     | 0    | 0      |
| Carcinoma             | 0          | 0     | 0     | 0    | 0      |
| Lost to follow-up     | 1          | 0     | 0     | 0    | 0      |
| **ASC-US**            | Number     | 0    | 12    | 0    | 0      | 0   | 0   | 12    |
| p16/Ki67              | 4 (33%)    | 4 (33%) | 0      | 3 (67%) |
| CIN1                  | 4 (33%)    | 4 (33%) | 0      | 2 (67%) |
| CIN 2                 | 4 (33%)    | 4 (33%) | 1 (33%) | 2 (67%) |
| CIN3                  | 4 (33%)    | 4 (33%) | 0      | 2 (67%) |
| Carcinoma             | 0          | 0     | 0     | 0    | 0      |
| **ASC-H**             | Number     | 0    | 0     | 0    | 8      | 0   | 0   | 8     |
| p16/Ki67              | 0          | 0     | 6 (75%) | 0      | 6 (85.7%) |
| CIN1                  | 0          | 0     | 2 (40%) | 0      | 0 (0%) |
| CIN 2                 | 0          | 0     | 3 (20%) | 1 (75%) |
| CIN3                  | 0          | 0     | 3 (20%) | 5 (12.5%) |
| Carcinoma             | 0          | 0     | 0     | 0    | 1 (12.5%) |
| **LSIL**              | Number     | 0    | 0     | 0    | 7      | 1   | 8   | 8     |
| p16/Ki67              | 0          | 0     | 6 (75%) | 0      | 6 (85.7%) |
| CIN1                  | 0          | 0     | 2 (40%) | 0      | 0 (0%) |
| CIN 2                 | 0          | 0     | 3 (20%) | 1 (75%) |
| CIN3                  | 0          | 0     | 3 (20%) | 5 (12.5%) |
| Carcinoma             | 0          | 0     | 0     | 0    | 1 (12.5%) |
| **HSIL**              | Number     | 0    | 0     | 0    | 8      | 1   | 9   | 9     |
| p16/Ki67              | 0          | 0     | 6 (85.7%) | 0      | 9 (100%) |
| CIN1                  | 0          | 0     | 0 (0%)  | 1 (12.5%) | 9 (100%) |
| CIN 2                 | 0          | 0     | 0 (0%)  | 0      | 0      |
| CIN3                  | 0          | 0     | 0 (0%)  | 0      | 0      |
| Carcinoma             | 0          | 0     | 0 (0%)  | 9 (100%) | 0      |
| **SCC**               | Number     | 0    | 0     | 0    | 9      | 9   | 9   | 9     |
| p16/Ki67              | 0          | 0     | 6 (85.7%) | 0      | 9 (100%) |
| CIN1                  | 0          | 0     | 0 (0%)  | 1 (12.5%) | 9 (100%) |
| CIN 2                 | 0          | 0     | 0 (0%)  | 0      | 0      |
| CIN3                  | 0          | 0     | 0 (0%)  | 0      | 0      |
| Carcinoma             | 0          | 0     | 0 (0%)  | 9 (100%) | 0      |
| **Total**             | Number     | 492  | 15    | 12    | 5       | 11  | 10  | 536   |
| p16/Ki67              | 483 (33%)  | 3 (33%) | 4 (33%) | 2 (50%) |
| CIN1                  | 1 (33%)    | 1 (33%) | 1 (33%) | 2 (50%) |
| CIN 2                 | 1 (33%)    | 1 (33%) | 1 (33%) | 2 (50%) |
| CIN3                  | 0          | 0     | 0     | 0    | 0      |
| Carcinoma             | 0          | 0     | 0     | 0    | 0      |
both the methods (89.47%), while the NPV was 64.23% for conventional Pap cytology, against 92.86% for LBC. *P* value was significant in both cases, however, it showed better correlation with LBC (<0.0001) than conventional cytology (0.0002). However, the percentage of unsatisfactory smears was better in LBC (1.65%) while compared to conventional Pap smear (4.2%), as was the screening time.

Table 2 shows correlation of p16/Ki-67 dual stain with histopathological diagnoses of the corresponding cases. Any histopathological abnormality (CIN1, CIN2, or CIN3) was considered as positive histopathological correlation. The data demonstrate how the dual immunostain positivity percentage increased with advancing grades of epithelial abnormality: it reached from 33% in ASC-US to 100% in both HSIL and SCC categories.

A total of 35 cases (67%) were p16/Ki-67 dual stain positive among the 52 cases with epithelial abnormalities that also underwent biopsy later in the course of management. Among the ASC-US category [Figure 1], 5 cases (33%) showed positive immunostaining, all of which were found to be CIN2/CIN3 on histopathology, whereas 3 (20%) cases showed negative staining despite having CIN2/CIN3 lesions. Seven (47%) cases that were found to have either CIN1 or no dysplasia were also negative for the immunocytology.

In the LSIL category [Figure 2], 8 (67%) cases showed positive immunostaining, among which 7 were confirmed to have a diagnosis of CIN2/CIN3 and 1 had the diagnosis of CIN1 on histology. All 4 cases with negative immunostaining were found to have either CIN1 or no dysplastic changes in the corresponding histology.

Comparative values of sensitivity, specificity, PPV, NPV, and two-sided *P* value of the double immunostain in detecting significant histological lesion (CIN2/CIN3) in cases diagnosed as ASC-US and LSIL on LBC were calculated, and in our study, for ASC-US, the sensitivity and specificity of the dual immunostain in detecting CIN2/CIN3 were found to be 100 and 70%, whereas for LSIL the values were 87.5 and 100%, respectively.

**DISCUSSION**

In teens and young adults, cervical cancer is indeed rare; and HPV infection, although very common, is usually transient and shows spontaneous resolution within a few years. The progression of a productive high-risk HPV infection to the neoplastic process involves an array of changes in the viral gene expression, i.e., the initial expression of structural genes needed for productive infection changing into a state of viral oncogenic expression interfering with the host’s cell cycle, thus leading to a transforming infection. Even histologically diagnosed CIN2 and CIN3 lesions appear to have strong molecular heterogeneity and a large percentage of them seem to regress spontaneously, and only 30–50% of large CIN3 lesions actually progress to invasive cervical carcinoma over a long period of time. The dual immunostain we used here attempts to recognize those changes occurring in the host cell. The goal of the study was to ascertain the role of the p16/Ki-67 immunostain in identifying histologically significant lesions in the patients with cytological diagnosis of mild atypia.

| LBC finding     | p16/Ki-67 dual stain positive in cytology | Histopathological correlation |
|-----------------|------------------------------------------|--------------------------------|
| ASC-US (15 cases) | 5 (33%)                                  | 14 (93%)                       |
| ASC-H (5 cases)  | 3 (60%)                                  | 5 (100%)                       |
| LSIL (12 cases)  | 8 (67%)                                  | 11 (91%)                       |
| HSIL (10 cases)  | 10 (100%)                                 | 9 (90%)                        |
| SCC (10 cases)   | 10 (100%)                                 | 10 (100%)                      |
| Total (52 cases) | 35 (67%)                                  | 50 (96%)                       |

**Table 2: p16/Ki-67 dual immunostaining and histopathological findings in different cytological abnormalities on LBC**

Figure 1: A case of ASC-US. (a) Conventional cytology (MGG stain, x400); (b) Liquid based cytology showing cluster of atypical squamous cells of undetermined significance (Papanicolaou stain, x400); (c) Positive dual immunostaining (400x); (d) Histology of the same case showing CIN1 (H and E, x100)

Figure 2: A case of LSIL. (a) Conventional cytology (MGG stain, x400); (b) Liquid based cytology showing low grade squamous intraepithelial lesion (Papanicolaou stain, x400); (c) Positive dual immunostaining (400x); (d) Histology of the same case showing CIN2 (H and E, x100)
The categories of ASC-US and LSIL remain difficult and challenging entities in terms of clinical management. ASC-US presents further challenges regarding diagnosis as the diagnostic criteria, and reproducibility still remains debated.[5]

In our study, the prevalence of CIN2 or worse lesions in ASC-US patients was 53%. This is significantly higher than other studies which showed the prevalence to be 5–22%.[6,7] This may be due to the effects of the nature of the other studies: this was a highly focused symptomatic group of patients and not routinely screened population; other studies had also been screened with a prior conventional Pap smear, but lacked the effect of age stratification. Similarly, the prevalence of CIN2+ lesions in the patients with cytological diagnosis of LSIL was found to be also high and reached 58.3%. Other studies have found it to be around 9–30%.[8,9]

In our study, 67% of all women with epithelial abnormality showed positive staining with p16/Ki-67 dual immunostain. The results closely follow previously conducted studies.[10-12] Our study showed the dual immunostain to have a sensitivity and specificity that approached 100 and 70%, respectively, in detecting CIN2+ lesions in ASC-US patients. While the PPV was 62.5%, the NPV reached 100% in this population. Other studies have shown a wide range of sensitivity (64–98%) and specificity (43–81%) for the immunostain in detection of high-grade precursor lesions in the ASC-US population.[2,7,10,12]

Among women with cytological findings of LSIL, the sensitivity and specificity of the immunostain to detect high-grade intraepithelial precursor lesions were 87.50 and 100%, respectively. PPV was 100% in our study population. Possati-Resende found the sensitivity and specificity to be 69.6 and 75.3%, respectively, in their study.[13] Consistent results have been achieved by other researchers in their studies.[10,12-16]

Although our study was limited by a small population size, lack of follow-up, and a lack of availability of concurrent HPV testing, the strengths lay in a well-defined study population and a cross-sectional study design, a population in each of whom a histological diagnosis was available, a lack of bias due to concurrent HPV testing, and usage of same fresh specimen (from residual material preserved specimen for LBC) for cytology and immunostaining.

Cervical cancer is one of the few cancers with the best scope for screening and prevention. While cytology has been an established method for screening, the diagnostic protocols still need to be further standardized, especially in regards to management of diagnoses such as ASC-US and LSIL, where the risk of progress to malignancy is significant but largely unpredictable by morphology alone. Even high-grade dysplasia has inherent heterogeneity in terms of risk of progression into invasive cancer, their timeline, and invasiveness. The simultaneous expression of a proliferation associated protein (Ki-67) and a protein associated with tumor suppression (p16) should confer a high specificity detection of dysplastic cells in which the neoplastic process has been initiated.[12] A morphology-independent interpretation also opens up new avenues in automated evaluation in this field.[10]

This dual immunostain may potentially be a useful tool in the triage of the ASC-US and the LSIL group, as more studies are coming up showing a significant rate of immunostain positivity and a high sensitivity for detecting high-grade precursor lesions in patients with these cytological abnormalities. With the available data, it follows that the number of colposcopic referral could be reduced to half by use of the dual immunostain technique. In resource-poor countries, the tradeoff between the cost of immunostain, HPV testing, and colposcopic referral protocols needs to be explored to ascertain the optimal strategy.

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Conflicts of interest
There are no conflicts of interest.

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