

ORIGINAL ARTICLE

Comparison of the sensitivity and specificity of p16/Ki-67 dual staining and HPV DNA testing of abnormal cervical cytology in the detection of histology proven cervical intraepithelial neoplasia grade 2 and above (CIN 2+)

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Abstract

Introduction: Human papillomavirus (HPV) testing is used as a means of triaging cervico-vaginal smears with low grade squamous abnormalities or as part of co-testing with cytology. While HPV testing has a high sensitivity, it has a low specificity in detecting cervical intraepithelial neoplasia grade 2 and above (CIN 2+) leading to unnecessary colposcopy referrals. We investigate the accuracy of the p16/Ki-67 dual immunocytochemical stain in determining the presence of CIN 2+ lesions on histology and its potential as a superior biomarker for triage. **Methods:** Liquid based cervico-vaginal cytology specimens with squamous abnormalities and corresponding histology from 97 women with subsequent colposcopy and biopsy were included. The specimens were then subjected to the dual stain and Roche Cobas 4800 multiplex real time PCR HPV DNA testing. The sensitivity and specificity of the dual stain and HPV testing were calculated using CIN 2+ on histology as a reference standard. **Results:** The sensitivity and specificity of the dual stain in detecting histology proven CIN 2+ was 93.7% and 76.5% while HPV testing was 85.7% and 14.7% respectively. Of the 44 women with ASCUS or LSIL on cytology, the dual stain also reduced the number of unnecessary colposcopy referrals from 27 to 7 when used as a triage marker compared to HPV testing. **Conclusion:** p16/Ki-67 dual stain was more sensitive and specific than HPV testing in determining the presence of CIN 2+ on histology. It could triage low grade cervico-vaginal specimens more effectively and potentially help women avoid unnecessary colposcopies. Future studies are needed to further evaluate its role in cervical cancer screening programmes.

Keywords: p16/Ki-67, cervix, cytology, neoplasia, immunocytochemistry

INTRODUCTION

Cervical cancer screening has come a long way since the advent of the Papanicolaou (Pap) stained cervical smear. Many women have benefitted from cervical cancer screening programmes with early detection of cervical intraepithelial neoplasia (CIN) and consequent reduction of mortality from invasive squamous cell carcinoma (SCC), especially in countries with organized screening programmes.^{1,2} In recent years, human papillomavirus (HPV) DNA/RNA testing has been incorporated into screening programmes, first as a means of triaging Pap cytology test with low grade abnormalities such as atypical

squamous cells of undetermined significance (ASCUS),^{3,4} and subsequently as part of co-testing with the Pap test. Even more recently, HPV testing has been approved as a primary screening tool. While HPV testing has a high sensitivity which compensates for the lower sensitivity of the Pap test, it suffers from a lower specificity. In instances where patients have a positive HPV result but a low grade squamous abnormality on cytology, subjecting these patients to colposcopy often turns out to be unnecessary as many of them have only benign or low grade dysplastic changes of the cervix on histology. There is, therefore, a need for a test to better triage women with HPV positive and a low grade cytology result. In 2011,

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a p16/Ki-67 dual immunocytochemical stain was proposed as a marker that could effectively triage women with low grade squamous abnormalities on cervical cytology.⁵ By detecting both p16 and Ki-67 positivity in a single cell, the dual stain seeks to identify the presence of a transforming infection and its associated high grade dysplasia. We aim to test the effectiveness of this dual stain in triaging women with ASCUS/LSIL on cervical smear for colposcopy, by assessing the sensitivity and specificity of this stain in identifying women with CIN grade 2 and above (CIN 2+) on subsequent histology and to compare the results of this dual stain test with HPV testing in identifying women with CIN 2+. We also hypothesized that the dual stain would be superior to the HPV test in preventing false positive referrals for colposcopy.

MATERIALS AND METHODS

Study design

This is a comparative retrospective study conducted at the cytopathology department of a tertiary hospital in Singapore. The eligible samples were obtained from the gynaecological service in our hospital either as part of routine screening for cervical cancer or as part of a diagnostic workup in patients with gynaecological symptoms and each sample underwent testing using the dual stain, HPV testing and histology analysis.

Only those ThinPrep liquid based cytology (LBC) cervico-vaginal specimens with squamous abnormalities ranging from ASCUS to squamous cell carcinoma were set aside from August 2014 to July 2015. The specimens of patients who underwent colposcopy and cervical/vaginal biopsy as a result of the abnormal smear were enrolled into the study and subjected to p16/Ki-67 dual immunocytochemical staining (CINtec PLUS) and HPV testing. Patients with subsequent hysterectomy were also enrolled as these patients also had a cervical or vaginal histology specimen that could act as reference/"gold" standard for the squamous abnormality seen on LBC specimens. The decision for colposcopy or hysterectomy was made on clinical grounds by the managing gynaecologist. LBC specimens were only enrolled into the study when there was a histological specimen available for comparison. In patients with more than one histology specimen, the first histology specimen obtained after the cervico-vaginal smear was used in the analysis. In total, 97 cases were collected. Ethics approval for the study was obtained from

a centralised institutional review board with a waiver of patient consent as all the specimen samples were anonymised. Funding for the dual stain and HPV testing was provided by a grant under the institution's Pathology Academic Clinical Programme.

p16/Ki-67 immunocytochemistry (CINtec PLUS/ dual stain) and HPV testing

For the p16/Ki-67 dual stain using the CINtec PLUS kit⁵, the residual ThinPrep vial leftover from the earlier cytology test was used to prepare a dual stained slide. The presence of 1 or more cervical epithelial cells showing within the same cell brown cytoplasmic and a red nuclear staining, indicative of p16 and Ki-67 co-expression, was defined as a positive result, regardless of morphologic interpretation. Cases without any double-immunoreactive cells were considered negative. The slides were evaluated by a pathology resident and a pathologist separately, with both having no prior knowledge of the cytology, HPV testing result or histology of the case. Cases with discrepant dual stain result were evaluated together to arrive at a consensus decision. A 2-day period of training by the manufacturer was provided prior to the slide evaluation.

For HPV testing, the residual material in the vial was sent for Roche Cobas 4800 multiplex real time PCR HPV test to look for the presence of HPV types 16, 18 or any of the other 12 high risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68).

Statistical analysis

Results were analysed using statistical software SPSS for Windows, Version 16. Using the histology results as the reference standard, we calculated the sensitivity and specificity of the following 3 modalities in the detection of high grade dysplasia: p16/Ki-67 dual stain, HPV testing by Cobas for all high risk type HPV i.e. HPV 16, 18 plus 12 other high risk types (henceforth abbreviated as HR-HPV) and HPV testing for HPV 16 and 18 only (abbreviated as HPV 16/18). A true positive test result would identify patients with CIN grade 2 or worse (CIN 2+) on histology while a true negative result would identify patients with CIN grade 1 or normal histology, as we aim to determine which test is superior in identifying patients who really need colposcopy (that is the group of patients with CIN 2+). We also plotted receiver-operating characteristics (ROC) curves using the method of

TABLE 1: Number of cases positive for p16/Ki-67 dual stain, HR-HPV, HPV 16/18 and number of CIN 2+ on histology categorised according to the cytology result

Cytology	No.	No. with CIN2+ on histology	No. with p16/Ki-67 dual stain (+)	No. with HR-HPV (+)	No. with HPV 16/18 (+)
ASCUS	17	6	7	14	9
LSIL	27	8	13	23	11
ASC-H	6	4	4	4	2
HSIL	39	37	36	35	22
SCC	8	8	7	7	7
Total	97	63	67	83	51

ASCUS: atypical squamous cell of undetermined significance, LSIL: low grade squamous intraepithelial lesion, HSIL: high grade squamous intraepithelial lesion, ASC-H: atypical squamous cell, a high grade lesion cannot be excluded, SCC: squamous cell carcinoma

successive parallelograms⁶ to identify the test that would provide optimal discrimination. Estimates were considered statistically significant for two-tailed values of $P < 0.05$.

RESULTS

The ages of the patients ranged from 24 to 88 years old (mean 45.9; median 42). The interval between the time of collection of the LBC specimen from the patient and the reporting of reference histology ranged from 0 to 272 days (mean 48.7 days; median 28 days). Of the 97 LBC specimens, 88 were from the cervix while 9 from vagina. The results of the cytology, together with the number of CIN 2+ that were proven on subsequent histology are tabulated in Table 1. The number of LBC specimens that were positive for the dual stain and HPV testing are also included in the table for comparison with the number of CIN 2+ proven on histology. The 97 corresponding histological specimens included colposcopic cervical/vaginal biopsies, cervical loop electrosurgical excision procedure (LEEP) specimens, cervical cone biopsies as well as 4 hysterectomy specimens. Of these specimens, 7

were negative for dysplasia, 27 had CIN 1, 16 had CIN 2, 35 had CIN 3 and 12 had squamous cell carcinoma.

The sensitivity and specificity of all 3 modalities in detecting CIN 2+ are tabulated in Table 2, and their positive and negative predictive values are summarised in Table 3. The sensitivity and specificity of the investigative modalities when considering only cytology specimens with ASCUS or LSIL are tabulated in Table 4, and their positive and negative predictive values are summarised in Table 5. Table 6 shows a detailed breakdown of the number of positive and negative tests for dual stain, HR-HPV and HPV 16/18 categorised according to the histology results in ASCUS and LSIL cases. For sensitivity and specificity of the various tests, we examined the ROC curves and found that there was a left shift for the dual stain when used to detect CIN 2+ on histology (Fig. 1), indicating high sensitivity and specificity. When detecting CIN 2+ in ASCUS and LSIL cases (n = 44), the dual stain again showed high sensitivity and specificity with a left shift of the ROC curve (Fig. 2). This observation was supported by the area under the ROC curve

TABLE 2: Sensitivity and specificity of all 3 investigative modalities in detecting CIN 2+ on subsequent histology

Type of investigation	Sensitivity (%)	Specificity (%)	AUC ^a	P-value
p16/Ki-67	59/63 (93.7)	26/34 (76.5)	0.851	0.000
HR-HPV	54/63 (85.7)	5/34 (14.7)	0.502	0.956
HPV 16 or 18	33/63 (52.4)	16/34 (47.1)	0.497	0.958

^aAUC, area under the ROC curve

TABLE 3: Positive and negative predictive values of all 3 investigative modalities in detecting CIN 2+ on subsequent histology

Type of investigation	Positive predictive value (%)	Negative predictive value (%)
p16/Ki-67	59/67 (88.1)	26/30 (86.7)
HR-HPV	54/83 (65.1)	5/14 (35.7)
HPV 16 or 18	33/51 (64.7)	16/46 (34.8)

TABLE 4: Sensitivity and specificity of p16/Ki-67 dual stain and HPV DNA testing in detecting CIN 2+ in cytology specimens with ASCUS or LSIL

Type of investigation	Sensitivity (%)	Specificity (%)	AUC ^a	P-value
p16/Ki-67	13/14 (92.9)	23/30 (76.7)	0.848	0.000
HR-HPV	12/14 (85.7)	5/30 (16.7)	0.512	0.845
HPV 16 or 18	5/14 (35.7)	15/30 (50.0)	0.429	0.387

^aAUC, area under the ROC curve.

TABLE 5: Positive and negative predictive values of p16/Ki-67 dual stain and HPV DNA testing in detecting CIN 2+ in cytology specimens with ASCUS or LSIL

Type of investigation	Positive predictive value (%)	Negative predictive value (%)
p16/Ki-67	13/20 (65.0)	23/24 (95.8)
HR-HPV	12/37 (32.4)	5/7 (71.4)
HPV 16 or 18	5/20 (25.0)	15/24 (62.5)

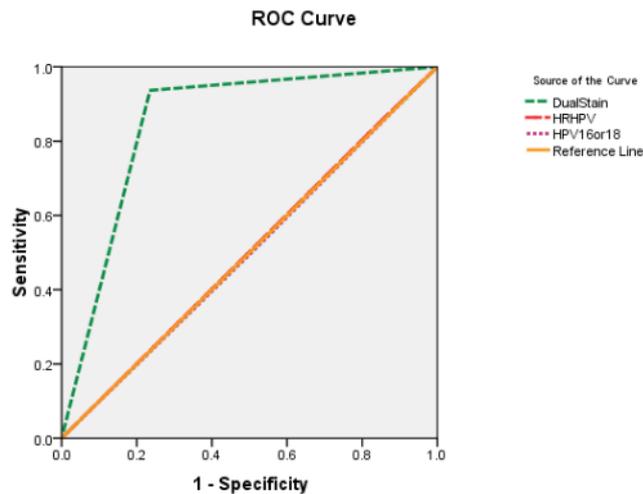


FIG. 1: ROC curves of the various tests in detecting CIN 2+ in all 97 cases. A left shift is seen in the dual stain, indicating optimal discrimination compared to HPV testing for any of the 14 high risk subtypes (HR-HPV) or for HPV 16 or 18 alone

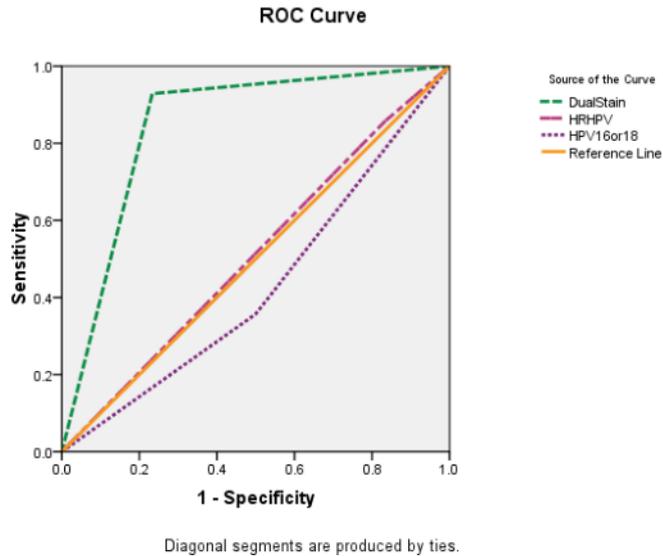


FIG. 2: ROC curves of the various tests in only the 44 ASCUS and LSIL cases. Again, a left shift is seen only in the dual stain and not in HPV testing

values and by significant ‘p’-values (Tables 2 and 4).

In our study, there were 11 cases of ASCUS with CIN 1 or lesser degree of abnormality (CIN 1-) on histology (Table 5). According to our institutional HPV genotyping and cytology co-testing management recommendations (Fig. 3), Pap tests with ASCUS that are positive for HR-HPV on co-testing would be referred for colposcopy. Of the 11, 8 were tested positive for HR-HPV, meaning that all 8 women would have undergone colposcopy even with a CIN 1- on histology. On the other hand, if the dual stain was used to triage Pap tests with ASCUS result, only 1 woman who was tested positive would undergo colposcopy. Among the 8 women tested positive for HR-HPV, 6 of them were positive for either HPV 16 or 18 or both (HPV 16/18). This means that even when the

presence of HPV type 16 or 18 was used to triage for colposcopy, 6 women would still have to undergo colposcopy. For the 27 women with LSIL on cytologic smear, 19 were CIN 1- on histology while 8 were CIN 2+. According to our institutional co-testing recommendations, all these cases would be referred to colposcopy regardless of the HPV result. Of the 19 women, only 6 were positive for the dual stain, meaning that 13 out of the 19 women could avoid a colposcopy if the dual stain were to be used as a triage tool. In summary, among the 30 women with ASCUS/LSIL on cytology and CIN 1- on histology, using the presence of any high risk HPV to triage these women would subject 27 of them to unnecessary colposcopy compared to 7 if the dual stain was used. Using the presence of only HPV 16 or 18 would still subject 25 women to colposcopy.

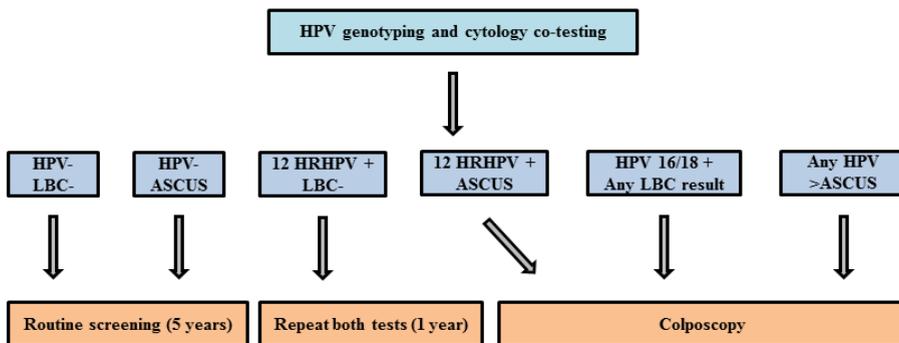


FIG. 3: HPV genotyping and cytology co-testing management recommendations; LBC – Liquid based cytology

TABLE 6: Number of cases positive for p16/Ki-67 dual stain, HR-HPV and HPV 16/18 categorised according to the cytology and histology

	No.	Histology	No.	p16/Ki-67 dual stain		HR-HPV		HPV 16/18	
ASCUS	17	Negative	4	(+)	0	(+)	2	(+)	2
				(-)	4	(-)	2	(-)	2
		CIN1/HPV	7	(+)	1	(+)	6	(+)	4
				(-)	6	(-)	1	(-)	3
		CIN2+	6	(+)	6	(+)	6	(+)	3
				(-)	0	(-)	0	(-)	3
LSIL	27	Negative	1	(+)	0	(+)	1	(+)	1
				(-)	1	(-)	0	(-)	0
		CIN1/HPV	18	(+)	6	(+)	16	(+)	8
				(-)	12	(-)	2	(-)	10
		CIN2+	8	(+)	7	(+)	6	(+)	2
				(-)	1	(-)	2	(-)	6
ASC-H	6	Negative	2	(+)	0	(+)	2	(+)	2
				(-)	2	(-)	0	(-)	0
		CIN1/HPV	0	(+)	0	(+)	0	(+)	0
				(-)	0	(-)	0	(-)	0
		CIN2+	4	(+)	4	(+)	2	(+)	0
				(-)	0	(-)	2	(-)	4
HSIL/SCC	47	Negative	0	(+)	0	(+)	0	(+)	0
				(-)	0	(-)	0	(-)	0
		CIN1/HPV	2	(+)	1	(+)	2	(+)	1
				(-)	1	(-)	0	(-)	1
		CIN2+	45	(+)	42	(+)	40	(+)	28
				(-)	3	(-)	5	(-)	17

DISCUSSION

The management of patients with low grade squamous abnormalities such as ASCUS and LSIL on cervical smears has long been a challenge. While HPV testing is a sensitive way to detect CIN 2+, it is not specific because the test cannot definitively differentiate between a transient infection, an early persistent infection that may develop to CIN 2+, or a prevalent CIN 2+ disease.⁷ Furthermore, in women with LSIL, the rates of infection for HPV are reported to be in the range of 80% to 85%,⁸ which renders the use of HPV testing as a triage for further management ineffective. The introduction of the p16/Ki-67 dual immunocytochemical stain in 2011 has generated much interest. This is due to the sound biologic model on which it is based and the encouraging results of initial studies.^{5,9,10} By detecting co-expression of both p16 (a cell cycle inhibitor protein) and Ki-

67 (a marker of cell proliferation) in a single squamous cell, this test seeks to identify cases in which a transforming infection has taken place. In transforming infections, the HPV E7 protein binds to the retinoblastoma protein in the squamous cell, leading to dysregulation of the cell cycle and paradoxical expression of both cell cycle inhibition and proliferation proteins. The presence of a transforming infection seems to be a more specific indicator of high grade dysplasia in the cervix, as evident from observations from the initial studies.^{5,9,10} This is consistent with our current understanding of the pathway of disease progression from CIN to invasive squamous cell carcinoma.

We found higher sensitivity of the dual stain when compared with HPV testing (93.7% vs. 85.7%). While many studies seem to show that HPV testing is more sensitive than p16/Ki-67 dual stain in detecting CIN 2+ disease,¹¹⁻¹³ our

findings indicate otherwise. Testing for HPV 16 or 18 only as a means to predict CIN 2+ on subsequent histology had increased specificity compared to HPV testing which includes other high risk subtypes. However, it showed a significant decrease in sensitivity. Dual stain was more specific than HPV testing (76.5% vs. 14.7%) in detecting CIN 2+ on histology, consistent with other studies in the existing literature.^{11,12,14} As a result, the use of the dual stain would be able to reduce the number of unnecessary colposcopies in women with ASCUS and LSIL. This is in concordance with two other studies which showed that there was a reduction in colposcopy referrals when the dual stain was used compared to HPV testing.^{15,16}

All these findings seem to indicate that the dual stain is a superior biomarker that can better predict the presence of CIN 2+ in patients with ASCUS or LSIL when compared to HPV co-testing with cytology. The superior sensitivity of the dual stain compared to HPV testing also raises the possibility of using the dual stain as a primary screening test with cytology as a second line triage, instead of HPV and cytology co-testing or using HPV as a primary screening tool with cytology for triage. Future large scale prospective studies will be needed to determine the best management algorithm that incorporates the dual stain and its role in cervical cancer screening.

One challenge that we encountered in the initial use of the dual stain was in the interpretation of the stain especially when staining was weak or with a substantial background artefact, whereby care was needed in identifying a true positive staining of a cell (Fig. 4). This was especially challenging in cases where very few positive cells with weak staining were seen. The fact that only one cell is required to call a case positive certainly leaves no room for error. The challenge was compounded in cases with very few positive cells and low grade cytology where the result of the dual stain was most at stake. This could possibly explain the varying indices of sensitivity (ranging from 64% to 98%) and specificity (ranging from 43% to 81%) in the existing literature.^{5,16,17} Certainly, an over conservative approach will lead to a reduced sensitivity of the stain. This was highlighted in one study¹¹ where the authors postulated that the low sensitivity of the dual stain in their study could be attributed to the difficulty in discriminating a weak cytoplasmic p16 staining from a background reaction, and

they classified the reaction as negative. Also, they expressed difficulty in classifying the reaction in three-dimensional cellular groups or metaplastic cells. While we acknowledge that these challenges are real, the training we received from the manufacturer in interpretation of the dual stain was very helpful and the results of our study showed that the dual stain had high discriminative value in the hands of trained personnel involved in this study. Of note, two studies also showed that the dual stain could be implemented with little training and that it was highly reproducible,^{18,19} lending support to the fact that this is not an overly difficult stain to use.

Another issue that arises with the use of the dual stain is the management of patients with dual stain positivity but without CIN 2+ on colposcopy. False positive results aside, the presence of cells that are dual stain positive but without CIN 2+ on histology may simply indicate that the patient already has a transforming HPV infection but has yet to progress to high grade dysplasia. One study showed that women with a positive dual stain result had a higher risk of developing a high grade lesion after 1 year of follow-up compared to dual stain negative women (23% vs. 3%).²⁰ Indeed, more prospective studies are required to determine the extent of follow up needed for these patients.

The strengths of our study include the presence of histology to confirm the presence or absence of disease in all cases and that the interpreters of the dual stain were blinded to the cytology, HPV testing and histology results. Limitations of our study include the retrospective acquisition of cases used for analyses, the lack of significant follow up in our cases, a selection bias by enrolling cases only where histology follow up was available leading to an enrichment of cases with more severe disease and the relatively small number of cases compared to the number of cervico-vaginal cytology specimens that are handled in nationwide cervical cancer screening programmes. As a result of the latter two points, the study population is not reflective of a true screening population.

In conclusion, the p16/Ki-67 dual stain had high sensitivity and specificity in predicting CIN 2+ in patients with abnormal Pap smears, compared to HPV testing. Using the dual stain to triage patients with ASCUS or LSIL could reduce the rate of colposcopy referrals significantly, compared to current HPV and cytology co-testing management algorithms. It is our belief that the

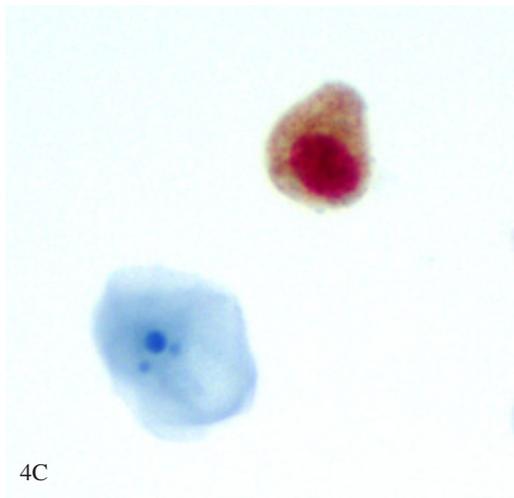
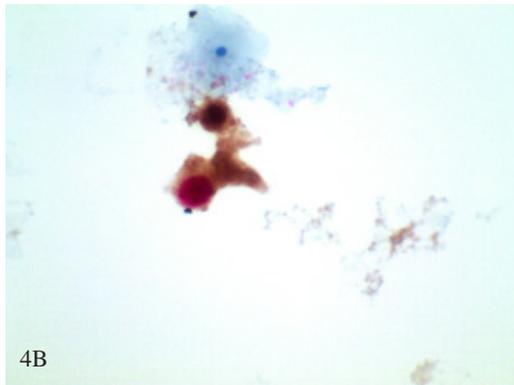
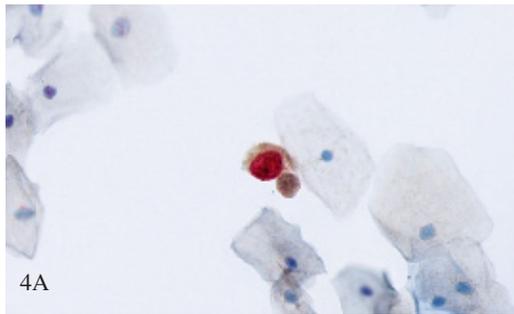


FIG. 4A-C: Photomicrographs of cells that exhibit positive staining for both p16 (brown) and Ki-67 (red) of the dual stain. In all three cases, there is definite staining of the cytoplasm and nucleus. In addition, the cytoplasm and nucleus are in the same plane and can be attributed to the same cell. The biopsy in all three cases revealed CIN 2+

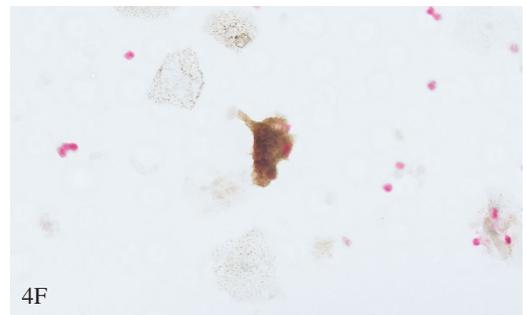
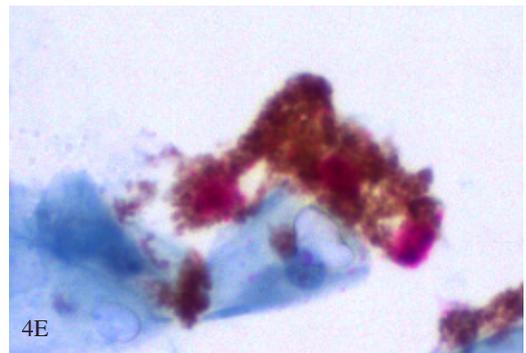
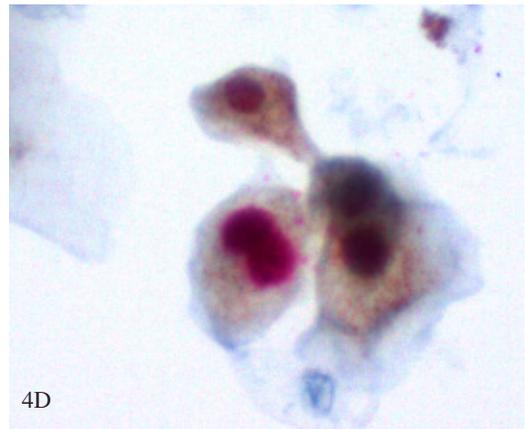


FIG. 4D-F: In comparison, these cells show either a cytoplasmic blush or non-specific granular staining for p16 and are regarded as negative. The biopsy revealed CIN 1 in all three cases

value of the dual stain in detecting high grade squamous abnormalities cannot be ignored and future studies should be done to further evaluate the cost effectiveness of using this biomarker in the larger schema of cervical cancer screening.

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