p16^{INK4a} Immunocytochemistry in Liquid-Based Cytology Samples in Equivocal Pap Smears

Added Value in Management of Women with Equivocal Pap Smear

Joseph Monsonego, M.D., **Giuseppe Pollini,** M.D., **Maria José Evrard,** M.Sc., **Patrice Sednaoui,** M.D., **Laura Monfort,** M.D., **Dominique Quinzat,** M.Sc., **Roger Dachez,** M.D., and **Kari Syrjänen,** M.D., Ph.D., F.I.A.C.

Objective

To test whether p16^{INK4a} immunocytochemistry (ICC) in liquid-based cytology (LBC) is useful with colposcopy in abnormal Pap smears.

Study Design

A series of 248 women with abnormal Pap smear were analyzed for oncogenic (HR) human papillomavirus (HPV) types using the Hybrid Capture II assay and for p16^{INK4a} expression using ICC on cervical samples in PreservCyt liquid media. Colposcopic and loop electrosurgical excision procedure (LEEP) cone biopsy were the gold standard.

In management of ASCUS, p16^{INK4a} ICC is clearly more specific than HCII, significantly improving the specificity and PPV of colposcopy.

Results

p16^{INK4a} ICC did best as predictor of high-grade squamous intraepithelial lesion, with OR 12.18 (2.72–54.57) (p = 0.0001), showing 88.2% sensitivity (SE), 61.9% specificity (SP), 14.6% positive predictive value (PPV) and 98.6% negative predictive value (NPV). In sorting discrepant cases, p16^{INK4a} ICC results in 100% SE and 100% NPV in detecting cervical intraepithelial neoplasia

From the Alfred Fournier Institute, Paris, France; and Department of Oncology and Radiotherapy, Turku University Hospital, Turku, Finland.

Dr. Monsonego is Scientific Director, Alfred Fournier Institute.

Dr. Pollini is Researcher, Alfred Fournier Institute.

Drs. Evrard and Montfort are Gynecologists, Alfred Fournier Institute.

Dr. Sednaoui is Colposcopist, Alfred Fournier Institute.

Ms. Quinzat is Junior Researcher, Alfred Fournier Institute.

Dr. Dachez is Director of Pathology, Alfred Fournier Institute.

Dr. Syrjänen is Professor, Department of Oncology and Radiotherapy, Turku University Hospital.

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Address correspondence to: Joseph Monsonego, M.D., EUROGIN, 174 rue de Courcelles, 75017 Paris, France (jm@eurogin.com).

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(CIN)2 lesions among Pap+/biopsy– women. In atypical squamous cells undetermined significance (ASCUS) cytology, adding p16^{INK4a} ICC improves specificity of colposcopy from 27.3% to 81.8% and PPV from 42.8% to 71.4%. Best performance is obtained with p16INK4a ICC and colposcopy: 83.3% SE, 81.8% SP, 71.4% PPV and 90.0% NPV.

Conclusion

p16^{INK4a} is useful in sorting clinically relevant discrepant cases, and p16^{INK4a} ICC significantly improves SP and PPV of colposcopy in management of ASCUS cytology.
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The conventional Pap test has proven its efficacy as the only cost-effective means to reduce the incidence and mortality of cervical cancer in countries where organized screening programs have been implemented (e.g., in the Nordic countries).[1,2] In most other countries, the screening programs or opportunistic screening efforts have not been equally successful, however.[2-4] This has challenged the importance of this simple, cost-effective and accurate test, attempting to question the well-documented benefits of the Pap test as a diagnostic and screening tool.[5,6] The foundations of that criticism derives from the fact that the true character of the 2 most frequent cytologic abnormalities (low grade squamous cell lesion, low grade squamous intraepithelial lesion [LSIL] and atypical squamous cells undetermined significance [ASCUS]) is still not fully understood, making their management strategies controversial and ambiguous.[7-9]

This has prompted a vigorous testing of other diagnostic tools in management of women with abnormal Pap smear results (MAPS).[7-9] Recently, a variety of such tools have been extensively tested in large trials, and different algorithms and recommendations have been launched by several professional societies.[10-14] These potential triage tools include liquid-based cytology (LBC), cytology automation and testing for human papillomavirus (HPV), the oncogenic types of which are the single most important etiologic agents of cervical intraepithelial neoplasia (CIN) and cervical cancer.[15-21] The benefits and limitations (including the cost-effectiveness) of these different approaches have been extensively discussed in the recent literature.[17-22]

The last few years have witnessed a rapidly expanding interest in molecular markers, not only as research tools used to increase our understanding of the molecular mechanisms of cervical cancer,[23,24] but also with increasing intensity as potential screening tools and, most recently, as novel means to triage women with equivocal Pap smear results.[25-29] The most intensely studied of all of these molecular markers is p16^{INK4a}, and emerging evidence suggests its usefulness as an adjunct diagnostic and screening tool, readily applicable in both histologic and cytologic samples.[23,26,30-37] In normal cells, the activity of CDK4 and CDK6 is strictly regulated by several CDK inhibitors, one of which is p16^{INK4a}.[23,31,34] Because expression of p16^{INK4a} is regulated by a negative feedback from pRB, reduced or lost pRB function results in up-regulation of p16^{INK4a} expression.[16-18,23,31,34] Accordingly, inactivation of pRB through binding with E7 of the high-risk human papillomavirus (hr-HPV) types should result in up-regulated expression of p16^{INK4a}, and the latter could represent a specific biomarker of cells expressing HPV E7.[23,31,33,34] This in turn should have widespread implications in cervical cancer screening,[28,29,34] and this concept has been tested in several recent reports evaluating the role of p16^{INK4a} as a marker of hr-HPV types,[23,31,34,38-41] in diagnosis of CIN[31,42-45] and, more recently, also SIL cytology.[46-49] The value of p16^{INK4a} as a useful marker of hr-HPV, CIN and cervical cancer has been established in most of these studies.

We here extend these studies to testing the performance indicators of p16^{INK4a} immunocytochemistry (ICC) in MAPS.[7] More specifically, we compared p16^{INK4a} ICC with 2 other diagnostic tools (ThinPrep and Hybrid Capture II [HCII] assay) in detecting CIN 2/3 under 2 special situations (1) in management of women with equivocal Pap smear results (ASCUS and atypical squamous cells, unable to exclude high grade SIL [ASC-H]), as well as (2) in sorting out clinically relevant discrepant cases, where conventional tests give discordant results. The major aim was to assess whether inclusion of p16^{INK4a} ICC would be of added value in this type of real-life setting of a major colposcopy referral clinic.

Materials and Methods

Patients

In the present study, we examined 248 women referred for colposcopic examination due to an abnormal Pap smear in a colposcopy clinic in Paris, France. All women were examined in the Institute Alfred Fournier (IAF), during November and December 2005, by 2 certified colposcopists (J.M. and G.P.). The mean age of the women was 35.1 years (SD 10.4, range 17–70, median 32.8). The women had a Pap smear taken in different clinics in Paris and were referred for colposcopic examination to IAF. All women had a new

cervical cytology sample taken, and all were examined by colposcopy and cervical biopsy or treated by loop electrosurgical excision procedure (LEEP) cone.

Cytology

All women had a previous Pap smear taken within 2–3 months before their enrollment in the study (i.e., the referral Pap), performed by community physicians. These baseline smears were examined by cytologists in several laboratories in Paris and were not available for reexamination by the authors. The smears were classified according to the 2001 Bethesda System (TBS 2001), and these original diagnoses were the baseline referral Pap smear diagnoses. The following diagnostic categories were included: ASCUS, 98 cases; ASC-H, 18 cases; LSIL, 105 cases; and high grade squamous intraepithelial lesion (HSIL) 27 cases (n = 248).

In the referral clinic, a new cervical cytology sample was taken from all of the women. Cervical samples for LBC were collected by a specially designed sampling device, which was rinsed into an LBC medium, PreservCyt (ThinPrep liquid Pap vial) (Cytyc Corporation, Marlborough, Massachusetts, U.S.A.) and prepared for ThinPrep specimens, following the manufacturer's recommendations. This medium is also validated for use with the HCII for HPV testing.[30]

Colposcopy

After sampling for LBC and HPV DNA testing (separately), colposcopic examination of the cervix, vagina and vulva was performed for all patients by 2 colposcopists, using a jointly agreed upon protocol. Lesions in the transformation zone (TZ) were assessed by applying 5% acetic acid and iodine solution, under ×8–12 magnification. If colposcopy proved unsatisfactory, further exploration of the endocervix was systematically carried out under ×20 magnification using a Koogan speculum.[50] The International Federation for Cervical Pathology and Colposcopy nomenclature[51] was used to classify the colposcopic patterns as normal; abnormal TZ (ATZ) with minor changes (with or without features of HPV infection), suggesting low grade CIN (CIN 1); ATZ with major changes suggesting CIN 2/3; and cancer. For statistical analysis, colposcopic results were dichotomized as either normal or abnormal.

Biopsy Procedures. All 248 women underwent colposcopic examination, with biopsy or LEEP. LEEP cone biopsy was performed in cases with (1) Pap test showing HSIL and ATZ in colposcopy; (2) regardless of the Pap test result, if the ATZ was large (≥ 50% of TZ area); (3) an endocervical lesion and unsatisfactory colposcopy; or (4) ATZ and a squamocolumnar junction localized > 3 mm within the endocervix. Altogether, 42 women underwent treatment by LEEP cone, while the rest (n = 206) had a directed punch biopsy taken.

Histology

All biopsies were examined in 1 pathology laboratory in Paris (Laboratoire Claude-Levy) and reported by 1 pathologist (R.D.). Histologic assessments were made as blinded by the HPV DNA status. In classifying the biopsies and LEEP samples, the CIN terminology was adopted.[5,6,16] For simplicity, histology was graded as normal (including metaplasia), CIN (including flat condyloma) and CIN 2+ (including CIN 3 and cervical cancer). In calculating the performance characteristics of p16INK4a ICC, cytology, colposcopy and HCII assay, we analyzed the biopsies and LEEP samples separately, used as the gold standard, and in statistical calculations, different cutoff values were tested: CIN 1 and CIN 2/3.

p16INK4a ICC. ICC analysis for p16INK4a was performed on the same samples as prepared for LBC (ThinPrep). Only the slides with a sufficient amount of cells were subjected to ICC analysis. The ICC procedures were performed using the CINtec p16INK4a Cytology Kit (Dako Cytomation AS, Clostrup, Denmark), designed for use on cytology specimens prepared either in the conventional way or using LBC. Technical details of the test have been described in previous studies using this assay.[35,36,46,48,49] The visualization system of the CINtec p16INK4a Cytology Kit is based on an optimized polymer and reagent for cytology specimens. The product is delivered in a kit format in which all reagents have been through extensive validation and quality control in order for the kit to perform with the most consistent results.

Evaluation of the ICC Staining. Evaluation of the ICC staining was performed by screening the whole slide at lower magnification and controlling the staining at magnification of ×400. All positive cells were counted in whole slide, and p16 reaction was classified positive if nuclear or cytoplasmic immunostaining was clearly demonstrated. In statistical calculations, reactions to p16INK4a were considered as positive or negative and not graded any further.

Hybrid Capture II (HCII) in LBC Medium

Separate specimens for HCII test were collected into PreservCyt LBC media (ThinPrep liquid Pap vial), validated for use with the HCII assay, following the manufacturer's instructions. Specimens collected in PreservCyt medium were transported to a laboratory at 2–30°C. Before analysis, specimens may be stored at

room temperature for up to 21 days or at 2–8°C for up to 8 weeks. The HCII assay was performed according to the instructions of the manufacturer (Digene Co., Gaithersburg, Maryland, U.S.A.). In estimation of positive reactions, samples were considered positive if the relative light units/control were > 1.0.52 Only hr-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) were tested in these samples.

Statistical Analysis

Statistical analyses were performed using the SPSS (SPSS for Windows, 13.1, Chicago, Illinois, U.S.A.) and STATA (STATA/SE 9.1, StataCorp, College Station, Texas, U.S.A.) software packages. Frequency tables were analyzed using the χ^2 test, and the likelihood ratio (LR) statistics or Fisher's exact test (where appropriate) were used to assess the correlation between the categorical variables. OR and 95% CI were calculated where appropriate. Differences in the means of continuous variables between the groups were analyzed using nonparametric tests (Mann-Whitney). Performance indicators of $p16^{INK4a}$ ICC, LBC, conventional cytology, colposcopy and HPV testing in detection of the outcome variables (CIN 1 or CIN 2/3) were calculated using the conventional contingency tables for sensitivity, specificity, positive (PPV) and negative predictive value (NPV), with 95% CI based on the F-distribution (± 1.96 × SE). The agreement (reproducibility) of cytologic diagnosis was controlled by comparing the diagnosis of the referral

Table I *Results of the Diagnostic Tests as Related to Each Other and Cervical Biopsy*

						Diagnostic p16	
	Cytology					Positive	Negative
Outcome test	**N**	**ASC**	**ASH**	**LSIL**	**HSIL**	**No. %**	**No. %**
Cytology							
Normal						57 (35.6)	103 (64.4)
ASCUS						10 (58.8)	7 (41.2)
ASC-H						13 (52.0)	12 (48.0)
LSIL						8 (27.6)	21 (72.4)
HSIL						15 (88.2)	2 (11.8)
						p = 0.0001[a]	
p16							
Positive	55.3	9.7	12.6	7.8	14.6		
Negative	71.0	4.8	8.3	14.5	1.4		
	p = 0.0001						
HCII							
Positive	53.4	9.2	14.1	14.1	9.2	77 (47.2)	86 (52.8)
Negative	85.9	2.4	2.4	7.1	2.4	26 (30.6)	59 (69.4)
	p = 0.0001					p = 0.014	
Colposcopy							
Normal	84.1	3.7	1.2	8.5	2.4	31 (37.8)	51 (62.2)
Abnormal	54.8	8.4	14.5	13.3	9.0	72 (43.4)	94 (56.6)
Major change	42.3	9.6	19.2	9.6	19.2	36 (69.2)	16 (30.8)
			N/AB; p = 0.0001			N/AB; p = 0.415	
			MC/NMC; p = 0.0001			MC/NMC; p = 0.0001	
Biopsy							
Normal (metaplasia)	81.5	3.7	4.6	9.3	0.9	33 (30.6)	75 (69.4)
CIN 1 (flat condyloma)	58.3	10.0	8.3	21.7	1.7	16 (26.7)	44 (73.3)
CIN 2 and more	33.3	18.5	11.1	7.4	29.6	18 (66.7)	9 (33.3)
	p = 0.0001					p = 0.001	
LLETZ							
Normal (metaplasia)	83.3	0.0	16.7	0.0	0.0	4 (66.7)	2 (33.3)
CIN 1 (flat condyloma)	62.5	12.5	25.0	0.0	0.0	4 (50.0)	4 (50.0)
CIN 2 and more	35.7	3.6	28.6	7.1	25.0	21 (75.0)	7 (25.0)
	p = 0.01					p = 0.416	

AB = abnormal, MC = dichotomized (major change vs. no major change [NMC]); N = normal; N/AB, dichotomized (normal vs. abnormal).
[a]Fisher's exact test.

Pap smear with the current LBC cytology, using Cohen's κ and weighted κ (intraclass correlation coefficient). In all tests, values $p < 0.05$ were regarded as statistically significant.

Results

The results of the different tests are related to each other as shown in Table I. There was only a modest concordance between the referral Pap and the current LBC, Cohen's κ = 0.400 (95% CI 0.358–0.442) ($p = 0.028$), and only the LBC results are used in all calculations. Cytologic abnormality is significantly related to p16INK4a expression, albeit the relationship is not linear. HCII positivity increases with cytologic abnormality. Cytology also closely correlates with abnormal colposcopy and colposcopy with major changes. p16INK4a expression is also significantly related to HCII test result ($p = 0.014$) and abnormal colposcopy with both cutoffs ($p = 0.0001$). HCII result is significantly ($p = 0.0001$) related to cytologic and colposcopic abnormality. Histology was analyzed separately for cervical biopsies (n = 206) and LEEP cones (n = 42). In biopsy, histologic grade bears a significant correlation to LBC, HCII, and abnormal colposcopy and also with p16INK4a expression. Albeit showing a similar trend, due to the small number of LEEP samples, these correlations showed a lower statistical significance.

Table II summarizes the performance indicators of each test as predictors of different outcomes at various

test	HCII				
Positive		Negative		Colposcopy	
No. %		No. %	N	N/AB	MC
87 (54.4)		73 (45.6)	69 (43.1)	91 (56.9)	22 (13.8)
15 (88.2)		2 (11.8)	3 (17.6)	14 (82.4)	5 (29.4)
23 (92.0)		2 (8.0)	1 (4.0)	24 (96.0)	10 (40.0)
23 (79.3)		6 (20.7)	7 (24.1)	22 (75.9)	5 (17.2)
15 (88.2)		2 (11.8)	2 (11.8)	15 (88.2)	10 (58.8)
p = 0.0001[a]				p = 0.0001[a]	p = 0.0001
77 (74.8)		26 (25.2)	31 (30.1)	72 (69.9)	36 (35.0)
86 (59.3)		59 (40.7)	51 (35.2)	94 (64.8)	16 (11.0)
p = 0.014				p = 0.415	p = 0.0001
			45 (27.6)	118 (72.4)	49 (30.1)
			37 (43.5)	48 (56.5)	3 (3.5)
				p = 0.012	p = 0.0001[a]
45 (54.9)		37 (45.1)			
118 (71.1)		48 (28.9)			
49 (94.2)		3 (5.8)			
	N/AB; p = 0.012				
	MC/NMC; p = 0.0001[a]				
56 (51.9)		52 (48.1)	42 (38.9)	66 (61.1)	4 (3.7)
37 (61.7)		23 (38.3)	55 (91.7)	5 (8.3)	3 (5.0)
26 (96.3)		1 (3.7)	27 (100)	0 (0.0)	17 (63.0)
p = 0.0001				**p = 0.0001	**p = 0.0001
4 (66.7)		2 (33.3)	2 (33.3)	4 (66.7)	0 (0.0)
6 (75.0)		2 (25.0)	8 (100)	0 (0.0)	2 (25.0)
25 (89.3)		3 (10.7)	27 (96.4)	1 (3.6)	24 (85.7)
p = 0.342				**p = 0.001	**p = 0.0001

Table II *Predictors of Abnormal Cytology (ThinPrep), Abnormal Colposcopy and CIN at Different Test and Outcome Cutoff Levels*

Predictive test	Outcome cutoff	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	OR (95% CI) % (95% CI)	p Value
p16	Cytology: ASC-US	52.3 (41.4–63.0)	64.4 (56.4–71.8)	44.7 (34.9–54.8)	71.0 (62.9–78.3)	1.97 (1.16–3.35)	0.011
	Cytology: ASC-H	50.7 (38.6–62.8)	62.1 (54.6–69.3)	34.9 (25.8–44.9)	75.9 (68.1–82.6)	1.68 (0.96–2.94)	0.064
	Cytology LSIL	50.0 (34.9–65.1)	60.4 (53.3–67.2)	22.3 (14.7–31.6)	84.1 (77.2–89.7)	1.52 (0.80–2.90)	0.246
	Cytology HSIL	88.2 (63.6–98.5)	61.9 (55.3–68.2)	14.6 (8.4–22.9)	98.6 (95.1–99.8)	12.18 (2.72–54.57)	0.0001
	Major change, Colpo	53.8 (36.5–67.7)	65.8 (58.7–72.4)	29.4 (20.5–39.7)	84.3 (77.6–89.7)	4.33 (2.27–8.37)	0.0001
	CIN 1	55.4 (44.7–65.8)	68.9 (60.8–76.3)	53.1 (42.7–63.4)	70.9 (62.7–78.3)	2.76 (1.60–4.75)	0.0001
	CIN 2/3	70.9 (57.1–82.4)	68.7 (61.4–75.3)	40.6 (30.7–51.1)	88.7 (82.2–93.4)	5.34 (2.76–10.35)	0.0001
HCII	Cytology: ASC-US	86.3 (77.3–92.7)	45.6 (37.7–53.6)	46.6 (38.7–54.5)	85.8 (76.6–92.4)	5.31 (2.68–10.52)	0.0001
	Cytology: ASC-H	85.9 (75.6–93.0)	42.3 (34.9–50.0)	37.4 (29.9–45.3)	88.2 (79.4–94.2)	4.48 (2.15–9.32)	0.0001
	Cytology LSIL	82.6 (68.5–92.1)	38.1 (31.3–45.2)	23.3 (17.1–30.5)	90.6 (82.3–95.8)	2.92 (1.29–6.60)	0.005
	Cytology HSIL	88.2 (63.5–98.5)	35.9 (29.7–42.4)	9.2 (5.2–14.7)	97.6 (91.8–99.7)	4.20 (0.93–18.84)	0.061
	Major change, Colpo	94.2 (84.0–98.7)	41.8 (34.8–49.0)	30.0(23.1–37.7)	96.4 (90.0–99.2)	11.74 (3.53–38.99)	0.0001
	CIN 1	85.8 (77.0–92.2)	48.2 (39.9–56.7)	51.3 (43.1–59.4)	84.3 (74.7–91.3)	5.67 (2.89–11.09)	0.0001
	CIN 2/3	92.7(82.4–97.9)	43.7 (36.1–50.9)	33.1 (25.7–41.1)	95.1 (88.1–98.6)	9.77 (3.39–22.80)	0.0001
Cytology							
LSIL	Major change, Colpo	28.8 (17.1–43.0)	84.1 (78.3–88.9)	32.6 (19.5–48.0)	81.6 (75.6–86.7)	2.15 (1.06–4.39)	0.039
	CIN 1	26.0 (17.4–36.2)	86.2 (79.5–91.3)	54.5 (38.8–69.6)	64.7 (57.5–71.4)	2.20 (1.13–4.28)	0.025
	CIN 2/3	34.5 (22.2–48.5)	86.2 (80.4–90.9)	43.1 (28.3-58.9)	81.3 (75.1–86.5)	3.31 (1.65–6.66)	0.001
HSIL	Major change, Colpo	19.2 (9.6–32.5)	96.4 (92.8–98.5)	58.8 (32.9–81.5)	81.8 (76.2–86.5)	6.42 (2.31–17.86)	0.0001
	CIN 1	16.3 (9.4–25.4)	98.6 (95.1–99.8)	88.3 (63.6–98.5)	65.0 (58.3–71.2)	13.92 (3.10–62.49)	0.0001
	CIN 2/3)	27.2 (16.1–40.9)	98.9 (96.1–99.8)	88.2 (63.6–98.5)	81.8 (76.1–86.6)	33.75 (7.42–153.49)	0.0001

Colpo = colposcopy.

Table III *Discrepant Cases Sorted by Adjunct Tests*

Discrepancy or double negative	Cytology (LSIL cutoff) SE	SP	PPV	NPV	Adjunct test P16 (+/–) SE	SP	PPV	NPV
Pap–, ≥ CIN 2					90.9[1]	37.5	66.6	75.0
Pap+, biopsy–*					100.0[2]	77.7	33.3	100.0
Pap–, colpo HG					92.9[3]	66.6	86.7	80.0
Pap+, colpo–					00.0[3]	57.1	00.0	80.0
p16+, biopsy–*								
p16–, ≥ CIN 2*	NC[1]	NC	NC	NC				
p16+, Pap–								
p16–, Pap+								
HCII–, ≥ CIN 2*	NC[1]	NC	NC	NC	66.6[1]	100.0	100.0	50.0
HCII+, Pap–					82.5[3]	75.0	46.7	94.1
HCII–, ≥ LSIL					100.0[3]	71.4	33.3	100.0
p16+, HCII–	50.0[3]	90.9	33.3	95.2				
p16–, HCII+	31.8[3]	82.3	36.8	78.8				
HCI–, Pap–					50.0[3]	71.0	4.7	98.0
p16–, Pap–								
P16–, HCII–	00.0[3]	91.2	00.0	96.3				
P16–, colpo–	100.0[3]	91.6	20.0	100.0				
HCII–, colpo–	00.0[4]	94.2	00.0	97.0	100.0[4]	68.5	8.3	100.0
Pap–, p16–, HCII–								

[1]CIN3 cutoff, [2]HSIL cutoff, [3]CIN2 cutoff and [4]CIN1 cutoff.
*Includes both biopsy and LEEP.
NC = not computable; PAP+, LSIL or higher.

cutoff levels of cytology and histology (biopsy and LEEP separately). p16INK4a performs best as predictor of HSIL cytology (OR 12.18, 95% CI 2.72–54.57). Sensitivity and NPV of p16INK4a ICC increases with increasing CIN grade, being best for CIN 2/3 (p = 0.0001). HCII shows similar SE across the whole spectrum of cytologic abnormalities, while specificity (SP) decreases and NPV increases toward HSIL. HCII is also a sensitive predictor of CIN at the expense of declining SP and increasing NPV toward high-grade CIN 2/3. Due to the limited number of LEEP cases, these figures are different from those obtained when biopsies are used as the reference. Performance of cytology is dependent on the cutoff (i.e., LSIL or HSIL), the best indicators being established for HSIL as predictor of CIN 3 (OR 55.96, 95% CI 12.1–258.7), followed by those for CIN 2 (OR 33.75, 95% CI 7.42–153.49) (for combined biopsy and LEEP histology).

The performance indicators for LBC, HCII and p16INK4a ICC in management of discrepant cases are calculated in Table III. At the end, all possible combinations of double negatives are listed and analyzed in the same manner. Colposcopy is by far the single most useful test, showing 100% SE and 100% NPV in solving a wide variety of discrepant situations, including the most relevant ones (e.g., Pap–/CIN 2/3; HCII+/Pap–; and most of the double negatives). The clinically most relevant discrepancies include the following: (1) Pap+/biopsy– and Pap+/colpo–; (2) HCII+/Pap– and p16INK4a+/Pap–; and (3) HCII–/Pap– and p16INK4a–/Pap–. In the management of these, both p16INK4a and HCII tests can be helpful, but in a divergent manner, in part complementing each other. Accordingly, in Pap+/biopsy– cases, performing p16INK4a ICC results in 100% SE and 100% NPV in detecting CIN 2 lesions, while using HCII does the same in Pap+/colpo– cases. In category 2, p16INK4a ICC has a 82.5% SP and 94.1% NPV in detecting CIN 2 in HCII+/Pap– cases, while HCII is 91.6% SE and 95.2% NPV in detecting CIN2 in p16INK4a+/Pap– cases. When either HCII or p16INK4a is negative together with a negative Pap (category 3), either of these 2 tests will aid the correct detection of CIN 2, with 98% NPV but with different SE, SP and PPV. Similarly, when both HCII and colposcopy are negative, p16INK4a expression predicts CIN 1 (or higher) with 100% SE and 100% NPV.

The role of each test as triage tools of women with ASCUS or ASC-H cytology is summarized in Table IV. Both HCII and p16INK4a ICC performs inadequately, if used as stand-alone tests in management of women with ASCUS cytology. In this setting, HCII correctly identifies 5/6 and p16INK4a ICC 4/6 CIN 2/3 lesions among the ASCUS patients, with 83.3% and 66.7% SE. The latter is clearly more specific and also has a better PPV and NPV. Of all combinations, the best performance in management of ASCUS cy-

used							
HCII (+/–)				Colposcopy (N/AB)			
SE	SP	PPV	NPV	SE	SP	PPV	NPV
81.8[1]	00.0	52.9	00.0	100.0[1]	62.5	78.5	100.0
50.0[2]	27.7	7.1	83.3	50.0[2]	33.3	7.6	85.7
85.7[3]	16.6	70.5	33.3				
100.0[3]	28.5	16.6	100.0				
50.0[2]	41.8	3.8	94.7	50.0[2]	55.8	5.0	96.0
94.1[1]	14.2	72.7	50.0	94.1[1]	00.0	69.5	00.0
91.6[3]	55.5	40.7	95.2	100.0[3]	58.3	44.4	100.0
100.0[3]	29.4	36.8	100.0	85.7[3]	23.5	31.5	80.0
				NC[1]	NC	NC	NC
100.0[3]	46.8	33.3	100.0				
				100.0[3]	28.5	16.6	100.0
				100.0[3]	54.5	16.6	100.0
				95.4[3]	35.2	32.3	96.0
				100.0[3]	49.2	5.4	100.0
85.7[3]	50.5	11.1	98.0	100.0[3]	44.3	11.4	100.0
				100.0[3]	42.1	5.7	100.0
100.0[3]	50.0	4.0	100.0				
				100.0[3]	46.9	3.7	100.0

Table IV *p16^{INK4a} Immunocytochemistry and Other Tests in Management of Women with ASCUS Cytology*

Test and combination	Performance in detecting CIN 2/3 lesions			
	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
ASCUS	24.0 (9.4–45.1)	92.4 (86.7–96.1)	35.3 (14.2–61.7)	87.5 (81.2–92.3)
ASCUS, HCII	83.3 (35.8–99.6)	9.1 (0.2–41.2)	33.3 (11.8–61.6)	50.0 (1.3–98.7)
ASCUS, p16^{INK4a}	66.7 (22.3–95.6)	54.5 (23.4–83.2)	44.4 (13.7–78.8)	75.0 (34.9–96.8)
ASCUS. colposcopy	100.0 (54.1–100)	27.3 (6.0–60.9)	42.8 (17.7–71.1)	100.0 (29.2–100)
ASCUS, HCII, p16^{INK4a}	83.3 (35.9–99.6)	54.5 (23.4–83.2)	50.0 (18.7–81.3)	85.7 (42.1–99.6)
ASCUS, colposcopy, HCII	83.3 (35.8–99.5)	36.4 (10.9–69.2)	41.7 (15.2–72.3)	80.0 (28.4–99.4)
ASCUS, colposcopy, p16^{INK4a}	83.3 (35.9–99.6)	81.8 (48.2–97.7)	71.4 (29.0–96.3)	90.0 (55.5–99.7)
Colposcopy (alone)[a]	98.1 (90.2–99.9)	41.2 (33.9–48.7)	33.5 (26.3–41.4)	98.6 (92.9–99.9)

Only women in these 2 categories are included in analysis.
[a]Whole series (shown here only for comparison).

tology is obtained when p16^{INK4a} ICC is combined with colposcopy, showing 83.3% SE, 81.8% SP, 71.4% PPV and 90.0% NPV (OR 22.50, 95% CI 1.60–314.56). This combination is markedly more specific than colposcopy with the HCII test and also has significantly higher PPV and NPV.

Discussion

To improve the accuracy and cost-effectiveness of CIN diagnosis particularly in cases with equivocal cytology, a variety of other diagnostic tools have been extensively tested during the past several years. These include automated cytology, LBC and HPV detection assays using HCII, or other newly introduced commercial tests (e.g., Roche AMPLICOR HPV test [Roche Molecular Diagnostics, Basel, Switzerland]).[15-21,53] More recently, the use of molecular markers as potential tests for triaging women with equivocal Pap results smear has been suggested.[23-29,31] Of the several potential markers reported in the recent literature, the most intensely studied molecular marker is p16^{INK4a}.[23,26,30-37] The rationale is that inactivation of pRB through binding with E7 of the hr-HPV types results in up-regulated expression of p16^{INK4a}, and the latter should represent a specific biomarker of cells expressing HPV E7, that is, CIN and cervical cancer cells.[23,31,33,34] This concept has been tested in several recent reports evaluating the role of p16^{INK4a} as a marker of hr-HPV types[23,31,34,38-41] in diagnosis of CIN,[31,32,42-45] as well as proving its applicability in Pap smears.[36,37,46-49,54-56] In several studies, p16^{INK4a} has been shown to be a specific marker of CIN and hr-HPV type,[23,31,32,34,38-45] but expression of this protein was of no prognostic value in cervical cancer or in predicting the clearance of hr-HPV after treatment of CIN.[31]

In the present study, we extended the analysis to testing the performance indicators of p16^{INK4a} ICC (applied in LBC samples) in management of women with abnormal Pap smear results. In a management setting, test specificity is more important, whereas in screening, a more sensitive test would be preferable. More specifically, we compared p16^{INK4a} ICC with 2 other diagnostic tools (ThinPrep and HCII assay) in detecting CIN 2/3 under 2 special circumstances: (1) in management of women with equivocal Pap smear results (ASCUS and ASC-H), as well as (2) in sorting out clinically most relevant discrepant cases, where other diagnostic tests give discordant results. To avoid any verification bias, all patients underwent colposcopy and punch biopsy, which was used as the gold standard technique. We also separately analyzed the 42 cases, where LEEP histology was used as the gold standard (Tables I and II). The limited number of LEEP samples did not markedly affect the performance indicators when histology was used as the reference; the biopsy series (n = 206) thus closely reflecting the combined histology (n = 248) data.

Technically, p16^{INK4a} ICC was readily applicable in the LBC sample collected for ThinPrep cytology, thus confirming the recent experience of others.[36,37,46-49,54-56] In our series of 248 women referred for abnormal Pap test, p16^{INK4a} ICC was closely ($p = 0.0001$) related to cytologic abnormality in ThinPrep, being most prevalent among ASC-H and HSIL cases (Table I). This intimate link between p16^{INK4a} and HSIL has been reported in the recent studies in which this technique was applied in Pap smears.[30,36,37,54-56] Interestingly, p16^{INK4a} expression was markedly less frequent (27.6%) in LSIL than in ASC-H and HSIL, being almost identical to the figures (25%) reported by Trunk et al[35] and those (21%) of Bose et al,[36] who also confirmed the high prevalence among ASC-H cases. These authors speculated that only a minority of LSIL cases might progress on to HSIL and p16^{INK4a} might be useful for triaging these patients for closer follow-up.[36] Accordingly, this would suggest that also ASC-H cytology showing a frequent (52%) p16^{INK4a} expression would represent cases at high risk for progression, which indeed was

the case in this series, in which CIN 2/3 was significantly more frequent among women with ASC-H than among LSIL cytology, 46% and 14.8%, respectively (Table I). On the other hand, the vast majority of LSIL cytology will eventually disappear, without ever progressing to high grade CIN. Indeed, using p16INK4a ICC in triage of ASCUS and LSIL cases should be helpful in distinguishing women at increased risk for true cervical disease, more accurately than does HCII assay.

In this study, p16INK4a expression was also significantly (p = 0.0001) related to the grade of cervical lesion in the biopsy and LEEP, being most frequent in CIN 2/3 lesions (Table I). This link to CIN grade was equally as strong as that of HCII positivity, albeit HPV positivity was more common than p16INK4a expression in all grades of CIN. As could be anticipated from this, p16INK4a and HCII test results were also closely correlated, but with less statistical power (p = 0.014). This is consonant with other recent studies failing to establish an intimate relationship between p16INK4a expression and HCII positivity in cervical cytology samples.[30,36,37] On the other hand, in studies in which p16INK4a staining has been applied to CIN biopsies, this marker usually shows a significant linear relationship to CIN grade.[31,32,42-45] Only a few studies exist in which p16INK4a expression in cytology has been correlated with concurrent biopsies.[54,56] Ekalaksananan et al[54] studied p16INK4a expression and HCII in Pap smears and biopsies, disclosing that all of the p16-positive cases of squamous metaplasia, CIN 2/3 and squamous cell cancer were hr-HPV-positive. In another study, Yoshida et al[56] analyzed a series of 98 cervical lesions, of which 16 demonstrated marked discrepancies between the cytologic and histologic diagnoses, in both their p16INK4a expression and hr-HPV detection. The most feasible explanation for this failure to establish equally close association between p16INK4a expression and CIN grade when the technique is performed in biopsies on one hand[31,32,42-45] and in cytologic samples on the other hand,[54,56] is the imperfect correlation between cervical cytology and biopsy. This is well illustrated in the present study, in which problematic are the histologic correlates of ASCUS and LSIL cytology, despite the significant overall correlation between ThinPrep and biopsy (p = 0.0001) (Table I).

Thus, when performed on the same cytologic (ThinPrep) samples, p16INK4a ICC can be anticipated to give the best correlation to cytologic abnormality as reported before[30,36,37,54-56] and also confirmed in our series (Table I). This is also well illustrated by the performance indicators calculated for the different tests (p16INK4a ICC, HCII, LBC) in detecting different outcome measures (SIL, CIN) (Table II). Not unexpectedly, HCII and colposcopy are the most sensitive tests in detecting CIN 2/3, whereas LBC with HSIL cutoff is by far the most specific test, being in alignment with most of the studies reported.[10-21] Cytology also had the best PPV (88.2%), whereas HCII showed the highest NPV (95.1%) in detecting CIN 2/3 (combined biopsy and LEEP). Compared with these figures, p16INK4a ICC performance was more modest, when the biopsy (or LEEP) was used as the gold standard. When HSIL was used as the outcome measure, however, p16INK4a ICC shows 88.2% SE, 61.9% SP, 14.6% PPV, and 98.6% NPV, detecting HSIL with OR 12.18 (95% CI 2.72–54.57) (Table II). These figures are almost identical to those of HCII, but the predictive power (OR) of the latter is much lower. Of the diagnostic tests used, colposcopy (major changes as cutoff) and HSIL cytology and are the most powerful tests, predicting CIN 2/3 (combined biopsy and LEEP) with OR = 56.29 (95% CI 22.79–139.00) (data not included in table), and OR = 33.75 (95% CI 7.42–153.49), respectively. These are followed by HCII with OR 9.77 (95% CI 3.39–22.80) and p16INK4a ICC, with OR = 5.34 (95% CI 2.76–10.35). Thus, neither HCII nor p16INK4a ICC can reach the overwhelming predictive power of cytology and colposcopy, which complement each other, colposcopy being more sensitive and cytology more specific. Also, the profile of p16INK4a ICC and HCII is different; despite its higher SE and NPV, HCII is less specific than p16INK4a ICC and has a lower PPV.

Thus, it seemed feasible to assess how these different tests perform in solving clinically relevant discrepant cases, that is, situations in which any of the 2-test combinations give discordant results (Table III). Although colposcopy (in this specialized clinic) is the single most useful test in solving a wide variety of discrepant situations, both p16INK4a and HCII tests can be helpful as well, but in a divergent manner and complementing each other. Of all possible discrepant cases, those being Pap+/biopsy– or Pap+/colpo– are among the clinically most relevant ones. Thus, in Pap+/biopsy– cases, performing p16INK4a ICC results in detection of CIN 2/3 lesions with 100% SE and 100% NPV, and using HCII does the same in Pap+/colpo– cases. In practice, asking p16INK4a ICC in the LBC sample seems a viable option in such cases, and a positive test strongly suggests a high-grade lesion, not detected in the biopsy. The same would be achieved by adding HCII test to triage Pap+ women with negative colposcopy. In cases with HCII+/Pap– or p16INK4a+/Pap–, p16INK4a ICC has a 82.5% SP in detecting CIN 2, while HCII is 91.6% SE and 95.2% NPV, respectively. Furthermore, in HCII–/Pap– or p16INK4a–/Pap– double negatives, either of these tests will aid the correct detection of CIN 2, with 98%

NPV but with different SE, SP and PPV. Similarly, when both HCII and colposcopy are negative, $p16^{INK4a}$ expression predicts CIN with 100% SE and 100% NPV. In this respect, the profile of HCII seems to be very similar in $p16^{INK4a}$–/colpo– lesions, raising the question about the feasibility of combining these tests.

Prompted by recently published data,[26,30,31,36,48,54-56] we finally analyzed the role of $p16^{INK4a}$ ICC alone or combined with HCII (and colposcopy) in management of women who are referred for colposcopy due to equivocal (ASCUS) cytology (Table IV). Both HCII and $p16^{INK4a}$ ICC performs inadequately, if used as stand-alone tests in this setting. Although HCII is more sensitive, $p16^{INK4a}$ ICC is clearly more specific and has better PPV and NPV. Similar results have been reported by other recent studies,[26,30,32,36,48,54-56] strongly advocating the inclusion of $p16^{INK4a}$ ICC as an adjunct biomarker in both MAPS, triage and screening. In the present management setting, the best performance is obtained, when $p16^{INK4a}$ ICC is combined to colposcopy, showing 83.3% SE, 81.8% SP, 71.4% PPV and 90.0% NPV. Thus, adding $p16^{INK4a}$ ICC in the diagnostic repertoire significantly improves the specificity of colposcopy in detecting CIN 2/3 lesions among ASCUS patients, from 27.3% up to 81.8%. At the same time, also PPV increases from 42.8% to 71.4% (Table IV). No such advantage is obtained by using HCII assay instead; showing equal sensitivity, this (HCII + colpo) combination is markedly less specific (36.4%) and has a lower PPV (41.7%) than colposcopy with $p16^{INK4a}$ ICC. All measures increasing the specificity of colposcopy are of potential clinical relevance, but the feasibility and cost-effectiveness of $p16^{INK4a}$ ICC in management of women with ASCUS cytology remain to be elucidated.

To conclude, when performed in the ThinPrep sample, $p16^{INK4a}$ ICC as a single test in MAPS setting does not exhibit remarkable performance in detecting CIN 2/3 in the biopsies or LEEP specimens. $p16^{INK4a}$ ICC performs best as a predictor of HSIL, showing 88.2% SE, 61.9% SP, 14.6% PPV and 98.6% NPV. It seems to be a useful test also in sorting out clinically relevant discrepant cases, e.g., those being (1) Pap+/biopsy–, (2) Pap+/colpo–, (3) HCII+/Pap–, and (4) HCII–/Pap– double negatives. In management of ASCUS, $p16^{INK4a}$ ICC is clearly more specific than HCII, significantly improving the specificity and PPV of colposcopy. Indeed, the best performance in managing ASCUS patients is obtained when colposcopy is combined with $p16^{INK4a}$ ICC. Although technically feasible to apply in the LBC samples, $p16^{INK4a}$ ICC is a potential adjunct tool in management of women with ASCUS cytology as well as in triage of such women for colposcopy. This test does not give any added value in management of ASC-H, however; and its performance in a screening setting remains to be fully elucidated in another type of study design.

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References

1. Hakama M: Screening for cervical cancer: Experience from the Nordic Countries. *In* New Developments in Cervical Cancer Screening and Prevention. Edited by E Franco, J Monsonego. Oxford, Blackwell Science, 1997, pp 190–199
2. Miller AB, Nazeer S, Fonn S, Brandup-Lukanow A, Rehman R, Cronje H, Sankaranarayanan R, Koroltchouk V, Syrjänen K, Singer A, Onsrud M: Report on Consensus Conference on Cervical Cancer Screening and Management. Int J Cancer 2000;86:440–447
3. Monsonego J: HPV infections and cervical cancer prevention: Priorities and new directions. Gynecol Oncol 2005;96:830–839
4. Ferlay J, Bray F, Pisani P, Parkin DM: GLOBOCAN 2002: Cancer incidence, mortality and prevalence worldwide. IARC Cancer Base No. 5, version 2.0. Lyon, IARC Press, 2004
5. Koss LG: Cervical (Pap) smear: New directions. Cancer 1993; 71:1406–1412
6. Koss LG: Performance of cytology in screening for precursor lesions and early cancer of the uterine cervix. *In* New Developments in Cervical Cancer Screening and Prevention. Edited by E Franco E, J Monsonego. Oxford, Blackwell Science, 1997, pp 147–150
7. Syrjänen KJ: Management of abnormal PAP smears (MAPS): Implications of terminology used in cytopathology. J Lower Genital Tract Dis 2000;4:217–223
8. Solomon D, Schiffman M: Have we resolved how to triage equivocal cervical cytology? J Natl Cancer Inst 2004;96:250–251
9. Cox JT: The clinician's view: Role of human papillomavirus testing in the American Society for Colposcopy and Cervical Pathology Guidelines for the management of abnormal cervical cytology and cervical cancer precursors. Arch Pathol Lab Med 2003;127:950–958
10. Arbyn M, Buntinx F, Van Ranst M, Paraskevaidis E, Martin-Hirsch P, Dillner J: Virologic versus cytologic triage of women with equivocal Pap smears: A meta-analysis of the accuracy to detect high-grade intraepithelial neoplasia. J Natl Cancer Inst 2004;96:280–293
11. American College of Obstetricians and Gynecologists: Practice bulletin: Clinical management guidelines for obstetrician-gynecologists: Human papillomavirus. No. 61, April 2005. Obstet Gynecol 2005;105:905–918
12. Ferris DG: The 2001 ASCCP management guidelines for cervical cytology. Am Fam Physician 2004;70:1866–1868
13. Wright TC Jr, Cox JT, Massad LS, Carlson J, Twiggs LB,

Wilkinson EJ: 2001 consensus guidelines for the management of women with cervical intraepithelial neoplasia. Am J Obstet Gynecol 2003;189:295–304

14. Monsonego J, Bosch FX, Coursaget P, Cox JT, Franco E, Frazer I, Sankaranarayanan R, Schiller J, Singer A, Wright TC Jr, Kinney W, Meijer CJ, Linder J, McGoogan E, Meijer C: Cervical cancer control, priorities and new directions. Int J Cancer 2004;108:329–333
15. Carozzi FM, Confortini M, Cecchini S, Bisanzi S, Cariaggi MP, Pontenani G, Raspollini MR, Sani C, Zappa M, Ciatto S: Triage with human papillomavirus testing of women with cytologic abnormalities prompting referral for colposcopy assessment. Cancer 2005;105:2–7
16. Syrjänen K, Syrjänen S: Papillomavirus Infections in Human Pathology. Chichester, J Wiley & Sons, 2000
17. Aubin, F, Pretet JL, Mougin C (editors): Papillomavirus Humains: Biologie et Pathologie Tumorale. Editions TEC and DOC, Paris, 2003
18. Campo S (editor): Papillomavirus Research: From Natural History to Vaccines and Beyond. UK, Caister Academic Press, Norwich, 2006
19. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ: Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003;348:518–527
20. Cliffort GM, Gallus S, Herrero R, Munoz N, Snijders PJF, Vaccarella S, Anh PTH, Ferreccio C, Hieu NT, Matos E, Molano M, Rajkumar R, Ronco G, de Sanjose S, Shin HR, Sukvirach S, Thomas JO, Tunsakul S, Meijer CJLM, Franceschi S: Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for research on Cancer HPV prevalence surveys: Pooled analysis. Lancet 2005;366:991–998
21. Legood R, Gray A, Wolstenholme J, Moss S: Lifetime effects, costs, and cost effectiveness of testing for human papillomavirus to manage low grade cytological abnormalities: Results of the NHS pilot studies. BMJ 2006;332:79–85
22. Melnikow J, Birch S: Human papillomavirus triage of atypical squamous cells of undetermined significance: Cost-effective, but at what cost? J Natl Cancer Inst 2006;98:82–83
23. von Knebel Doeberitz M: New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. Eur J Cancer 2002;38:2229–2242
24. Syrjänen KJ: Immunohistochemistry in assessment of molecular pathogenesis of cervical carcinogenesis. Eur J Gynaecol Oncol 2005;26:118–124
25. Ault KA, Allen HK, Phillips SL, Zimmerman MB, Klingelhutz AJ: Telomerase activity as a potential diagnostic marker for triage of abnormal Pap smears. J Low Genit Tract Dis 2005;9:93–99
26. Carozzi F, Cecchini S, Confortini M, Becattini V, Cariaggi MP, Pontenani G, Sani C, Ciatto S: Role of P16(INK4a) expression in identifying CIN2 or more severe lesions among HPV-positive patients referred for colposcopy after abnormal cytology. Cancer 2006;106:496–504
27. Branca M, Giorgi C, Santini D, Di Bonito L, Ciotti M, Costa S, Benedetto A, Casolati EA, Favalli C, Paba P, Di Bonito P, Mariani L, Syrjänen S, Bonifacio D, Accardi L, Zanconati F, Syrjänen K. Aberrant expression of a novel inhibitor of apoptosis (Survivin) is related to grade of cervical intraepithelial neoplasia (CIN), but does not predict virus clearance after cone or prognosis in cervical cancer. Am J Clin Pathol 2005;124:113–121
28. Padilla-Paz LA: Emerging technology in cervical cancer screening: Status of molecular markers. Clin Obstet Gynecol 2005;48:218–225
29. Cheng Q, Lau WM, Chew SH, Ho TH, Tay SK, Hui KM: Identification of molecular markers for the early detection of human squamous cell carcinoma of the uterine cervix. Br J Cancer 2002;86:274–281
30. Longatto Filho A, Utagawa ML, Shirata NK, Pereira SM, Namiyama GM, Kanamura CT, Santos Gda C, De Oliveira MA, Wakamatsu A, Nonogaki S, Roteli-Martins C, Di Loreto C, Mattosinho de Castro Ferraz Mda G, Maeda MY, Alves VA, Syrjanen K: Immunocytochemical expression of p16INK4A and Ki-67 in cytologically negative and equivocal pap smears positive for oncogenic human papillomavirus. Int J Gynecol Pathol 2005;24:118–124
31. Branca M, Ciotti M, Santini D, Di Bonito L, Giorgi C, Benedetto A, Paba P, Favalli C, Costa S, Agarossi A, Alderisio M, Syrjanen K: p16INK4A Expression is related to grade of CIN and high-risk human papillomavirus but does not predict virus clearance after conization or disease outcome. Int J Gynecol Pathol 2004;23:354–365
32. Benevolo M, Mottolese M, Marandino F, Vocaturo G, Sindico R, Piperno G, Mariani L, Sperduti I, Canalini P, Donnorso RP, Vocaturo A: Immunohistochemical expression of p16(INK4a) is predictive of HR-HPV infection in cervical low-grade lesions. Mod Pathol 2006;19:384–391
33. O'Neill CJ, McCluggage WG: p16 Expression in the female genital tract and its value in diagnosis. Adv Anat Pathol 2006;13:8–15
34. von Knebel Doeberitz M: New molecular tools for efficient screening of cervical cancer. Dis Markers 2001;17:123–128
35. Trunk MJ, Dallenbach-Hellweg G, Ridder R, Petry KU, Ikenberg H, Schneider V, von Knebel Döberitz M: Morphologic characteristics of p16INK4a-positive cells in cervical cytology samples. Acta Cytol 2004;48:771–782
36. Bose S, Evans H, Lantzy L, Scharre K, Youssef E: p16(INK4A) is a surrogate biomarker for a subset of human papilloma virus-associated dysplasias of the uterine cervix as determined on the Pap smear. Diagn Cytopathol 2005;32:21–24
37. Moore GD, Lear SC, Wills-Frank LA, Martin AW, Snyder JW, Helm CW: Differential expression of CDK inhibitors p16, p21cip1, p27kip1, and cyclin E in cervical cytological smears prepared by the ThinPrep method. Diagn Cytopathol 2005;32:82–87
38. Keating JT, Cviko A, Riethdorf S, Riethdorf L, Quade BJ, Sun D, Duensing S, Sheets EE, Munger K, Crum CP: Ki-67, cyclin E, and p16INK4 are complementary surrogate biomarkers for human papilloma virus-related cervical neoplasia. Am J Surg Pathol 2001;25:884–891
39. Keating JT, Ince T, Crum CP: Surrogate biomarkers of HPV infection in cervical neoplasia screening and diagnosis. Adv Anat Pathol 2001;8:83–92
40. Sano T, Masuda N, Oyama T, Nakajima T: Overexpression of p16 and p14ARF is associated with human papillomavirus infection in cervical squamous cell carcinoma and dysplasia. Pathol Int 2002;52:375–383
41. Agoff SN, Lin P, Morihara J, Mao C, Kiviat NB, Koutsky LA: p16(INK4a) expression correlates with degree of cervical neoplasia: A comparison with Ki-67 expression and detection of high-risk HPV types. Mod Pathol 2003;16:665–673

42. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, von Knebel Doeberitz M: Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 2001;92:276–284
43. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T: Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. Am J Pathol 1998;153:1741–1748
44. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T: Immunohistochemical overexpression of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia. Pathol Int 1998;48:580–585
45. Klaes R, Benner A, Friedrich T, Ridder R, Herrington S, Jenkins D, Kurman RJ, Schmidt D, Stoler M, von Knebel Doeberitz M: p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. Am J Surg Pathol 2002;26:1389–1399
46. Saqi A, Pasha TL, McGrath CM, Yu GH, Zhang P, Gupta P: Overexpression of p16INK4A in liquid-based specimens (SurePath) as marker of cervical dysplasia and neoplasia. Diagn Cytopathol 2002;27:365–370
47. Murphy N, Ring M, Killalea AG, Uhlmann V, O'Donovan M, Mulcahy F, Turner M, McGuinness E, Griffin M, Martin C, Sheils O, O'Leary JJ: p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. J Clin Pathol 2003;56:56–63
48. Nieh S, Chen SF, Chu TY, Lai HC, Fu E: Expression of p16INK4A in Papanicolaou smears containing atypical squamous cells of undetermined significance from the uterine cervix. Gynecol Oncol 2003;91:201–208
49. Bibbo M, Klump WJ, DeCecco J, Kovatich AJ: Procedure for immunocytochemical detection of p16ink4a antigen in thin-layer, liquid-based specimens. Acta Cytol 2002;46:25–29
50. Monsonego J, Javier Pintos J, Semaille C, Beumont M, Dachez R, Zerat L, Bianchi A, Franco E: HPV testing improves the accuracy of colposcopy in detection of cervical intraepithelial neoplasia. Int J Gynecol Cancer 2006;16:591–598
51. Walker P, Dexeus S, De Palo G, Barrasso R, Campion M, Girardi F, Jakob C, Roy M, Nomenclature Committee of the International Federation for Cervical Pathology and Colposcopy: International terminology of colposcopy: An updated report from the International Federation for Cervical Pathology and Colposcopy. Obstet Gynecol 2003;101:175–177
52. Howard M, Sellors J, Kaczorowski J, Lorincz A: Optimal cutoff of the Hybrid Capture II human papillomavirus test for self-collected vaginal, vulvar, and urine specimens in a colposcopy referral population. J Lower Genital Tract Dis 2004;8:33–37
53. Monsonego J, Bohbot JM, Pollini G, Krawec C, Vincent C, Merignargues I, Haroun F, Dachez R, Syrjänen K: Performance of the ROCHE AMPLICOR® human papillomavirus (HPV) test in detection of cervical intraepithelial neoplasia (CIN) in management of women with abnormal PAP smear. Gynecol Oncol 2005;99:160–168
54. Ekalaksananan T, Pientong C, Sriamporn S, Kongyingyoes B, Pengsa P, Kleebkaow P, Kritpetcharat O, Parkin DM: Usefulness of combining testing for p16 protein and human papillomavirus (HPV) in cervical carcinoma screening. Gynecol Oncol 2006;103:62–66
55. Sahebali S, Depuydt CE, Boulet GA, Arbyn M, Moeneclaey LM, Vereecken AJ, Van Marck EA, Bogers JJ: Immunocytochemistry in liquid-based cervical cytology: Analysis of clinical use following a cross-sectional study. Int J Cancer 2006;118:1254–1260
56. Yoshida T, Fukuda T, Sano T, Kanuma T, Owada N, Nakajima T: Usefulness of liquid-based cytology specimens for the immunocytochemical study of p16 expression and human papillomavirus testing: A comparative study using simultaneously sampled histology materials. Cancer 2004;102:100–108