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Chapter 4

Good performance of p16/Ki-67 dual-stained cytology for surveillance of women treated for high-grade CIN

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Abstract

Women treated for high-grade cervical intraepithelial neoplasia (CIN) are at risk of recurrent CIN Grade 2 or worse (rCIN2+). Currently, post-treatment monitoring is performed using cytology or cytology/high-risk (hr)HPV co-testing. This study aimed to evaluate the performance of p16/Ki-67 dual-stained cytology (p16/Ki-67) for post-treatment monitoring. Three hundred and twenty-three women treated for high-grade CIN in the SIMONATH study underwent close surveillance by cytology, hrHPV and DNA methylation marker testing up to 12 months post-treatment. Histological endpoints were ascertained by colposcopy with biopsy at 6 and/or 12 months. p16/Ki-67 dual-staining was performed on residual liquid-based cytology samples obtained at, or shortly before biopsy collection. Clinical performance estimates of cytology, hrHPV, p16/Ki-67 testing and combinations thereof for the detection of rCIN2+ were determined and compared to each other. Sensitivity of p16/Ki-67 for rCIN2+ (69.2%) was non-significantly lower than that of cytology (82.1%; ratio 0.84, 95% CI: 0.71–1.01), but significantly lower than that of hrHPV testing (84.6%; ratio 0.82, 95% CI: 0.68–0.99). Specificity of p16/Ki-67 for rCIN2+ (90.4%) was significantly higher compared to both cytology (70.8%; ratio 1.28, 95% CI: 1.19–1.37) and hrHPV testing (76.2%; ratio 1.19, 95% CI: 1.12–1.26). Overall, hrHPV testing showed very high sensitivity, along with a good specificity. When considering co-testing, combined p16/Ki-67/hrHPV testing showed rCIN2+ sensitivity comparable to cytology/hrHPV co-testing (87.2% vs. 89.7%; ratio 0.97, 95% CI: 0.92–1.03), but with significantly increased specificity (74.2% vs. 58.1%; ratio 1.28, 95% CI: 1.19–1.38). Thus, when considered in combination with hrHPV, p16/Ki-67 might be an attractive approach for surveillance of women treated for high-grade CIN.

What's new?

Normally the cell-cycle regulator p16 and the proliferation marker Ki-67 are not expressed in the same epithelial cell unless the cell is dysregulated after infection with a high-risk human papilloma virus (hrHPV). Here the authors used p16/Ki-67 dual-stained cytology in combination with hrHPV testing in women treated for high-grade intraepithelial cervical neoplasia (CIN2/3). They show that this test is as sensitive as conventional cytology/hrHPV co-testing to detect recurrence. However, it results in lower colposcopy referrals due to higher specificity and positive predictive value, making it an interesting approach to monitor women for recurrent CIN2/3.

Introduction

Women treated for high-grade cervical intraepithelial neoplasia Grade 2 and 3 (CIN2/3) are at risk of developing recurrent high-grade CIN (rCIN2+) for up to 10–20 years after treatment.[1,2] Because of this increased risk, close surveillance of these women is necessary. Preferably, this surveillance should be done with a test strategy with a high sensitivity. On the other hand, a strategy that has a high specificity as well is desirable to minimize overtreatment, which especially is important for women in reproductive age, as pregnancy outcomes are influenced by the proportion of cervix that is removed[3]. Surveillance strategies for women treated for high-grade CIN vary between countries. Currently, in many European countries women are monitored by cytology testing at 6, 12 and 24 months after treatment.[4,5] It has been shown that addition of high-risk human papillomavirus (hrHPV) testing to cytology, so-called co-testing, increases the sensitivity for rCIN2+.[1,6] Accordingly, Dutch Guidelines recommend cytology/hrHPV co-testing as a strategy for follow-up of women treated for high-grade CIN. When both cytology and hrHPV test-results are negative at 6 months post-treatment, testing at 12 months can be omitted without an increased rCIN risk[1]. However, the main disadvantage of cytology testing and cytology/HPV co-testing is the limited specificity for rCIN2+.[7–9] Therefore, alternative monitoring strategies are urgently needed.

Recently, cross-sectional studies have shown that p16/Ki-67 dual-stained cytology has an overall high sensitivity and specificity for detecting high-grade CIN.[10–13] Under physiologically normal conditions, overexpression of p16, a cell-cycle regulatory protein that induces cell-cycle arrest[14,15] and expression of proliferation marker Ki-67 are mutually exclusive and therefore not found in the same cervical epithelial cell. In fact, detection of p16/Ki-67 co-expression is a sign of hrHPV-induced deregulation of the cell cycle, which may be used as an indicator of a transforming hrHPV infection and the presence of high-grade CIN lesions.[12,13,16]

This study was set out to evaluate the performance of p16/Ki-67 dual-stained cytology relative to cytology and hrHPV testing for monitoring women treated for high-grade CIN.

Material and Methods

Study population

This study was conducted as a post hoc analysis within the prospective, multicenter SIMONATH study (Simplified Monitoring of Post-treatment CIN2/3 Women by Molecular Testing for HPV and Methylation Markers).[17] The SIMONATH study comprised post-treatment monitoring of women treated for CIN2/3 and was conducted in six participating outpatient clinics in The Netherlands. All women aged 18 years and older scheduled for a LLETZ (large loop excision of the transformation zone) procedure for treatment of a biopsy-confirmed high-grade CIN between April 2010 and June 2012 were asked to participate in the SIMONATH study. Women in whom the presence of a high-grade CIN was confirmed in the LLETZ specimen, who were treated directly without prior biopsy (see-and-treat procedure), could also be included in the study. The study was approved by the Medical Ethical Committee (METC) of VU medical center (METC-VUmc2009/285) and endorsed by all other participating clinics and registered in Dutch Trial Register (NTR1964). All participating women provided written informed consent. A total of 364 women treated for CIN2/3 were enrolled and follow-up at 6 and 12 months post-treatment.

Procedures

In the SIMONATH study, the performance of different biomarkers (i.e., cytology, hrHPV and methylation markers) was evaluated to monitor women for post-treatment disease to

optimize post-treatment management.[17] For this purpose, liquid-based cytology (LBC) samples were obtained prior to treatment using a Cervex-Brush (Rovers Medical Devices B.V., Oss, The Netherlands). Samples were collected and stored in Thinprep (Hologic, Malborough, MA) and used for hrHPV detection and methylation marker analysis. At 6 months follow-up, two cervical scrapes were obtained from each participating women. The first scrape was collected for cytology according to local hospital protocols. The second scrape was collected in ThinPrep for hrHPV testing and methylation marker analysis. According to protocol women were referred for colposcopy with biopsy at 6 months if one or more of the following conditions were fulfilled: (i) cytology ≥BMD (borderline or mild dyskaryosis) or ≥ASC-US (atypical squamous cells of undetermined significance according

to The Bethesda classification) and/or (ii) a positive hrHPV test and/or (iii) a positive methylation marker test. A total of 164 women had biopsy with histology outcome at 6 months. Of the women without post-treatment disease (rCIN2+) at 6 months

post-treatment, one cervical scrape was obtained and stored in ThinPrep at 12 months post-treatment for cytology, hrHPV and methylation marker analysis in the reference laboratory. After obtaining the cervical scrape, an exit-colposcopy with mandatory biopsy was performed. Women with post-treatment disease at either 6 or 12 months follow-up were treated according to national guidelines with a re-LLETZ or cervical conisation.[4,5]

Cytology slides were Pap-stained and scored according to the CISOE-A classification, which is easily translatable into the Bethesda 2001 classification.[18] Cytological results were grouped as normal, borderline or mild dyskaryosis (BMD), or moderate dyskaryosis or worse (>BMD).

DNA from cervical scrapes was isolated using magnetic beads (Macherey-Nagel, Germany) and an automated DNA extraction platform (Microlab Start; Hamilton Robotics, Switzerland), according to the recommendations of the manufacturer. Detection of hrHPV was performed using the clinically validated GP5+/6+-PCR with an enzyme immunoassay readout[19,20].

At colposcopy, biopsies were taken from the most suspicious areas and in case no suspicious lesion was present, a random biopsy was collected. Histological specimens were classified as normal, CIN Grades 1, 2 or 3, or as invasive cancer.[21] According to Dutch guidelines, uncertain cases were reviewed after p16 immunohistochemistry.[4]

Women were included in this post hoc study when a histological endpoint was reached, implying that a histological specimen was obtained and graded. Additionally, a LBC sample obtained at, or close before, the moment the histological endpoint was reached, had to be available for p16/Ki-67 staining. Also cytology and hrHPV test results from that same LBC sample should be present.

Cytology slides were prepared from residual LBC samples using a Thinprep 5000 processor (Hologic), after being stored at 48C for approximately 2 years (mean: 802 days; range: 452–1,313). After preparation slides were subjected to p16/Ki-67 dual staining after a maximum of 4 days (mean: 2 days; range:1–4). For p16/Ki-67 dual staining, as described previously[22], a commercial kit designed for simultaneous detection of p16 and Ki-67 in cervical cytology preparations was used (CINtec PLUS, Roche mtm Laboratories AG, Mannheim, Germany). Slides were subjected to p16/Ki-67 dual staining according to the manufacturer's instructions. The p16/Ki-67 dual-stained slides were reviewed by an experienced cytotechnologist blinded to all study data. Cells were considered positive when p16 and Ki-67 immunoreactivity were

revealed within the same cell (i.e., a cytoplasmic brown staining for p16, together with a nuclear red staining for Ki-67). The presence of at least one dual-stained cell was used as a cutoff to rate the sample as positive for CINtec PLUS. Additionally, analysis with cutoffs of 2 or more and 6 or more p16/Ki-67 dual-stain positive cells was performed.

Statistical analysis

Sample size calculation was performed based on the primary study protocol.[17] Sample size was set at 360, under the assumptions that 10% of women would present with ≥rCIN and 5% of women would withdraw from the study. The primary endpoint was post-treatment disease, defined as histological confirmed CIN2/3 after previous treatment. In January 2014, follow-up data of all women with post-treatment disease were verified from local hospital databases. Histological endpoint was assessed based on outcome of biopsies or, if classified worse, on outcome of LLETZ or conisation. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and colposcopy rates of p16/Ki-67 dual-stained cytology testing, cytology testing, hrHPV testing, cytology/hrHPV co-testing and p16/Ki-67/hrHPV co-testing for detection of rCIN3+ and rCIN2+ were calculated from cross-tabulation of test results with exact 95% confidence intervals (CIs). In case of co-testing, the result was considered positive if one or both of the combined tests showed a positive test result. Relative sensitivities (ratios of the sensitivity of one test to the sensitivity of another test) and relative specificities (ratios of the specificity of one test to the specificity of another test) for paired data were calculated with 95% CIs to enable comparison between tests.[23] A difference in sensitivity or specificity was considered significant if the 95% CI of the relative sensitivity or specificity was completely below or above one. PPVs and NPVs between tests were compared using the method of Leisenring et al[24]. Differences between colposcopy rates were calculated using the McNemar test. All statistical analyses were performed in IBM SPSS Statistics and the statistical software program R (version 3.0.1).[25]

Results

Study cohort characteristics

From a total of 364 women treated for CIN2/3 in the SIMONATH study, 41 women were excluded from further analysis in this study because of incomplete follow-up (Figure 1). In 26 of these women no exit-colposcopy with mandatory biopsy was performed and therefore no histological endpoint was reached. Reasons for not having an exit-colposcopy with biopsy were patients not showing up for colposcopy (n=13), no

colposcopy performed at follow-up visit (n=6), pregnancy (n=4), hysterectomy (n=1), emigration (n=1) and death (n=1). Another 15 women were excluded because of not having a follow-up cervical scrape (n=3), unavailability of liquid-based cytology (LBC) sample (n=10) or absence of hrHPV and/or cytology test result on the retrospective scrape (n=2). Ultimately, 323 women were included into this post hoc study. Thirty-five (10.8%) of them reached a histological endpoint at 6 months follow-up, and 288 women (89.2%) reached a histological endpoint at 12 months follow-up during exit-colposcopy with biopsy.

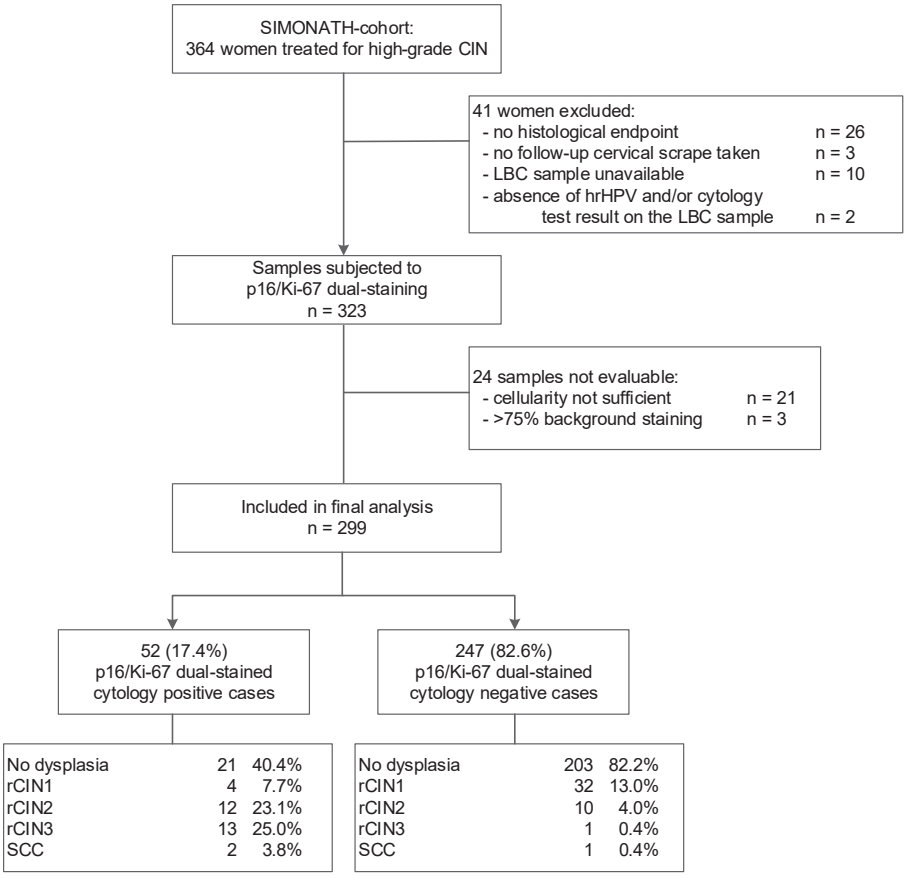

Figure 1. Flowchart of the SIMONATH-study design, including p16/Ki-67 dual-stained cytology test results.

n = number of women; LBC = liquid-based cytology; (r)CIN = (recurrent) cervical intraepithelial neoplasia; SCC = squamous cell carcinoma.

Residual LBC samples of these 323 post-treatment women were subjected to p16/Ki-67 dual-stained cytology testing; 24 (7.4%) of these cases were excluded from further analysis because the dual-stained slides could not be interpreted (Figure 1). Of these, 21 (87.5%) had insufficient cellularity and 3 (12.5%) had >75% background staining. A total of 299 women were used in the final analysis, of whom 52 (17.4%) tested positive for p16/Ki-67 dual-stained cytology (threshold ≥1 cell positive). The median age of these women was 35 years (range 19–59 years). Ninety-five (31.8%) women were hrHPV positive, of whom 59 (62.1%) had abnormal cytology (i.e., ≥BMD). Two hundred and four (68.2%) women were hrHPV negative, of whom 49 (24.0%) had ≥BMD cytology. In total, 3 (1.0%) squamous cell carcinomas (SCC), 14 (4.7%) rCIN3, 22 (7.4%) rCIN2 and 36 (12.0%) rCIN1 lesions were diagnosed. Two hundred and forty-four (74.9%) women did not show dysplasia in their biopsies.

Test performance characteristics

Test performance characteristics are shown in Table 1. Sensitivity of p16/Ki-67 dual-stained cytology for rCIN2+ (69.2%; 95% CI: 54.7–83.7%) was statistically not significantly different from that of cytology (82.1%; 95% CI: 70.0–94.1%; ratio 0.84, 95% CI: 0.71–1.01), but was significantly lower than that of hrHPV testing (84.6%; 95% CI: 73.3–95.9%; ratio 0.82, 95% CI: 0.68–0.99). However, specificity of p16/Ki-67 dual-stained cytology for rCIN2+ (90.4%; 95% CI: 86.8–94.0%) was significantly higher than that of cytology (70.8%; 95% CI: 65.2–76.3%; ratio 1.28, 95% CI: 1.19–1.37) and hrHPV testing (76.2%; 95% CI: 71.0–81.3%; ratio 1.19, 95% CI: 1.12–1.26). The positive predictive value (PPV) of p16/Ki-67 dual staining for rCIN2+ (51.9%) was significantly higher compared to both cytology (29.6%; $p<0.001$) and hrHPV testing (34.7%; $p<0.001$). The negative predictive value (NPV) was comparable between p16/Ki-67 dual staining and cytology (95.1% and 96.3%, respectively; $p<0.29$). The NPV of p16/Ki-67 (95.1%) tended to be lower compared to hrHPV testing (97.1%; $p<0.096$). Colposcopy rates based on p16/Ki-67 dual-stained cytology (17.4%) were significantly lower compared to colposcopy rates based on cytology (36.1%; $p<0.001$) and hrHPV testing (31.8%; $p<0.001$).

For rCIN3+ outcome, similar results were obtained. Differences in sensitivity rates between the tests for rCIN3+ were smaller than those at the rCIN2+ threshold; p16/Ki-67 dual staining showed a slightly lower sensitivity (88.2%; 95% CI: 63.6–98.5%) which did not reach statistical significance, neither when compared with cytology (94.1%; 95% CI: 71.3–99.9%; ratio 0.94, 95% CI: 0.83–1.06) nor to hrHPV testing (100.0%; 95% CI: 80.5–100.0%; ratio 0.88, 95% CI: 0.74–1.05). Specificity of p16/Ki-67 dual-stained cytology for rCIN3+ (86.9%; 95% CI: 82.9–90.8%), however, was significantly higher compared to both cytology (67.4%; 95% CI: 61.9–72.8%; ratio 1.29, 95% CI: 1.20–1.39)

Table 1. Clinical performance of p16/Ki-67 dual-stained cytology, cytology testing, hrHPV testing and combinations of these tests for detection of rCIN2+ and rCIN3+.

	n1/N1	Sensitivity	95% CI	n2/N2	Specificity	95% CI	PPV	95% CI	NPV	95% CI	Colpo Rate
p16/Ki-67											
rCIN2+	27/39	69.2%	(54.7-83.7%)	235/260	90.4%	(86.8-94.0%)	51.9%	(38.3-65.5%)	95.1%	(92.5-97.8%)	17.4%
rCIN3+	15/17	88.2%	(63.6-98.5%)	245/282	86.9%	(82.9-90.8%)	28.8%	(16.5-41.2%)	99.2%	(97.1-99.9%)	17.4%
Cytology											
rCIN2+	32/39	82.1%	(70.0-94.1%)	184/260	70.8%	(65.2-76.3%)	29.6%	(21.0-38.2%)	96.3%	(93.7-99.0%)	36.1%
rCIN3+	16/17	94.1%	(71.3-99.9%)	190/282	67.4%	(61.9-72.8%)	14.8%	(8.1-21.5%)	99.5%	(97.1-100.0%)	36.1%
hrHPV											
rCIN2+	33/39	84.6%	(73.3-95.9%)	198/260	76.2%	(71.0-81.3%)	34.7%	(25.2-44.3%)	97.1%	(94.7-99.4%)	31.8%
rCIN3+	17/17	100.0%	(80.5-100.0%)	204/282	72.3%	(67.1-77.6%)	17.9%	(10.2-25.6%)	100.0%	(97.3-100.0%)	31.8%
p16/Ki-67 and/or hrHPV											
rCIN2+	34/39	87.2%	(76.7-97.7%)	193/260	74.2%	(68.9-79.5%)	33.7%	(24.4-42.9%)	97.5%	(95.3-99.7%)	33.8%
rCIN3+	17/17	100.0%	(80.5-100.0%)	198/282	70.2%	(64.9-75.6%)	16.8%	(9.5-24.1%)	100.0%	(98.2-100.0%)	33.8%
Cytology and/or hrHPV											
rCIN2+	35/39	89.7%	(80.2-99.3%)	151/260	58.1%	(52.1-64.1%)	24.3%	(17.3-31.3%)	97.4%	(94.9-99.9%)	48.2%
rCIN3+	17/17	100.0%	(80.5-100.0%)	155/282	55.0%	(49.2-60.8%)	11.8%	(6.5-17.1%)	100.0%	(97.6-100.0%)	48.2%

hrHPV = high-risk human papillomavirus; rCIN2+(3+) = recurrent cervical intraepithelial neoplasia grade 2(3) or worse; n = number of cases; N = total number of cases; CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value.

Table 2. Clinical performance of p16/Ki-67 dual-stained cytology with different thresholds for detection of rCIN2+ and rCIN3+.

	n1/N1	Sensitivity	95% CI	n2/N2	Specificity	95% CI	PPV	95% CI	NPV	95% CI	Colpo Rate
p16/Ki-67 ≥1 pos. cell											
rCIN2+	27/39	69.2%	(54.7-83.7%)	235/260	90.4%	(86.8-94.0%)	51.9%	(38.3-65.5%)	95.1%	(92.5-97.8%)	17.4%
rCIN3+	15/17	88.2%	(63.6-98.5%)	245/282	86.9%	(82.9-90.8%)	28.8%	(16.5-41.2%)	99.2%	(97.1-99.9%)	17.4%
p16/Ki-67 ≥2 pos. cells											
rCIN2+	26/39	66.7%	(51.9-81.5%)	239/260	91.9%	(88.6-95.2%)	55.3%	(41.1-69.5%)	94.8%	(92.1-97.6%)	15.7%
rCIN3+	15/17	88.2%	(63.6-98.5%)	250/282	88.7%	(85.0-92.4%)	31.9%	(18.6-45.2%)	99.2%	(97.2-99.9%)	15.7%
p16/Ki-67 ≥6 pos. cells											
rCIN2+	19/39	48.7%	(33.0-64.4%)	250/260	96.2%	(93.8-98.5%)	65.5%	(48.2-82.8%)	92.6%	(89.5-95.7%)	9.7%
rCIN3+	11/17	64.7%	(42.0-87.4%)	264/282	93.6%	(90.8-96.5%)	37.9%	(20.3-55.6%)	97.8%	(96.0-99.5%)	9.7%

hrHPV = high-risk human papillomavirus; rCIN2+(3+) = recurrent cervical intraepithelial neoplasia grade 2(3) or worse; n = number of cases; N = total number of cases; CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value.

and hrHPV testing (72.3%; 95% CI: 67.1–77.7%, ratio 1.20, 95% CI: 1.13–1.28). PPV of p16/Ki-67 dual staining for rCIN3+ (28.8%) was significantly higher compared to cytology (14.8%; p<0.001) and hrHPV testing (17.9%; p<0.002), at comparable NPVs [99.2% for p16/Ki-67, compared to 99.5% (p<0.50) for cytology and 100.0% (p<0.16) for hrHPV testing].

When considering combined p16/Ki-67/hrHPV testing, a similar rCIN2+ sensitivity as in case of cytology/hrHPV co-testing was revealed (87.2% vs. 89.7%; ratio 0.97. 95% CI:

0.92–1.03), but at a significantly higher rCIN2+ specificity (74.2% vs. 58.1%; ratio 1.28, 95% CI: 1.19–1.38). Additionally, the PPV of p16/Ki-67/hrHPV co-testing for rCIN2+ was significantly higher compared to cytology/hrHPV co-testing (33.7% vs. 24.3%; p<0.001), at comparable NPV's (97.5% vs.97.4%; p<0.92). Therefore, the use of combined p16/Ki-67/hrHPV testing would lead to significantly lower colposcopy rates compared to cytology/hrHPV co-testing (33.8% vs.48.2%; p<0.001). As in this cohort hrHPV testing showed a sensitivity of 100.0% for rCIN3+, adding co-testing with cytology or p16/Ki-67 dual-stained cytology could not further improve the detection rate for rCIN3+. However, specificity of p16/Ki-67/hrHPV co-testing for rCIN3+ was significantly higher than of cytology/hrHPV co-testing (70.2% vs. 55.0%; ratio 1.28, 95% CI: 1.19–1.37). Also for rCIN3+, PPV of p16/Ki-67/hrHPV co-testing was significantly higher compared to cytology/hrHPV co-testing (16.8% vs. 11.8%; p<0.001), whereas the NPV was identical (100.0%) for both approaches.

Finally, we evaluated the clinical performance of p16/Ki-67 dual-stained cytology at thresholds of ≥2 and ≥6 dual-stain positive cells. As expected, increasing thresholds resulted in lower sensitivities and higher specificities (Table 2).

Discussion

The aim of this study was to evaluate the clinical performance of p16/Ki-67 dual-stained cytology for post-treatment monitoring of women previously treated for high-grade CIN. As women treated for high-grade CIN have a substantial risk of developing post-treatment disease, surveillance should preferably be done with a strategy with a high sensitivity. However, as mentioned before, a high specificity is desirable to prevent overtreatment with associated negative side effects. In our analysis, it appeared that compared to cytology and hrHPV testing, p16/Ki-67 dual-stained cytology had a consistently higher specificity for both rCIN2+ and rCIN3+, although with a slightly lower sensitivity.

Traditionally, cytology testing has been used for monitoring women treated for high-grade CIN. When considering sole testing, hrHPV testing herein showed a very high sensitivity for detection of rCIN2+, together with a good specificity. Based on these results, sole hrHPV-testing would be a suitable approach for surveillance of women treated for high-grade CIN. Over the last years, cytology/hrHPV co-testing is often recommended as a strategy for follow-up of women treated for high-grade CIN, to prevent missing CIN2+ lesions that might be negative for hrHPV.[1,4,26] For this reason, additional co-testing strategies have been evaluated. Compared to cytology/hrHPV co-testing, p16/Ki-67/hrHPV co-testing had a similar sensitivity for rCIN2+, but a significantly higher specificity. This high specificity translates into a significant decrease in the number of colposcopy rates. Therefore, when considering co-testing, this combination of p16/Ki-67/hrHPV tests would be an interesting alternative co-testing approach for follow-up of women treated for CIN2/3. Additionally, one out of three SCC cases was negative for p16/Ki-67 dual-stained cytology (Figure 1), but would have been identified using the combined p16/Ki-67 dual-stained cytology plus hrHPV testing approach.

This study is the first study evaluating the clinical performance of p16/Ki-67 dual-stained cytology for monitoring women previously treated for high-grade CIN. Previous studies on p16/Ki-67 dual-stained cytology were focusing on triage of HPV-positive women[12,22,27], on triage of women with ≥BMD (ASC-US) cytology[11,28,29] or on a colposcopy referral population.[10] Results of our study are mainly consistent with data of these studies showing that p16/Ki-67 dual-stained cytology has a similar or somewhat lower sensitivity and a higher specificity compared to hrHPV testing for the detection of CIN2/3.

The higher specificity of p16/Ki-67 dual staining compared to cytology and hrHPV testing is reflected in a higher PPV. PPV of p16/Ki-67 dual staining was significantly higher compared to cytology and hrHPV testing, which was also reflected by the significantly lower colposcopy rates based on p16/Ki-67. In addition, when p16/Ki-67 dual staining is combined with hrHPV testing a significantly higher PPV and lower colposcopy rates of p16/Ki-67/hrHPV co-testing were found compared to cytology/hrHPV co-testing. Moreover, all analyzed tests showed high NPVs, which is of great value for the surveillance of women previously treated for high-grade CIN.

For the analysis of p16/Ki-67 staining in this study and previous studies[22,27], a threshold of 1 or more p16/Ki-67 dual-stained positive cell was used. However, the assessment of dual-stained slides is still dependent on the review of slides by individual readers and therefore subject to some remaining reader variability.[30]

Aiming at increasing the reproducibility, we also analyzed the performance of p16/Ki-67 dual-stained cytology at higher thresholds. When raising the threshold of p16/Ki-67 dual-stained cytology, higher specificities with corresponding lower colposcopy rates were found. However, this resulted in lower sensitivities. As in a diagnostic setting of monitoring post-treatment CIN2/3, a test with high sensitivity is mandatory, increasing the threshold would not lead to an improved performance of p16/Ki-67 dual-stained cytology.

Strengths of this study are the large sample size and that nearly all women studied had a histological endpoint. Only 26 (7.1%) of women had neither exit-colposcopy nor biopsy, thereby they did not reach a histological endpoint and were excluded from our analyses. The alternative of including these women as having no evidence of CIN2+, yielded comparable test performance characteristics (data not shown). A limitation of this study, however, is that 7.4% of p16/Ki-67 dual-stained samples were not evaluable due to insufficient cellularity or background staining. An explanation for this might be that prior to preparation of the slide for p16/Ki-67 dual staining, the LBC samples were already used for cytology and molecular tests. A second explanation could be that for practical reasons, some LBC samples were obtained directly before colposcopy, thereby obtaining suboptimal scrapings due to the physician's attempt to avoid bleeding during colposcopy.[31] Another limitation is that LBC samples were stored for approximately 2 years prior to preparation of new cytology slides for p16/Ki-67 dual staining. We cannot fully exclude that the storage period affected sample cellularity. Previous studies evaluating the effect of storage time on p16 and p16/Ki-67 immunohistochemistry showed that a long storage time may lead to decreased and weakened staining of cells, resulting in an underestimation of the performance of dual-stained cytology.[11,32] Finally, our results are cross-sectional data based on 1 year of follow-up. Long-term follow-up data, at least until 24 months after treatment, would be of value for further evaluation of p16/Ki-67 dual-stained cytology, as this is common in Western countries.

In conclusion, sole hrHPV testing showed a good combination of sensitivity and specificity and would therefore be a suitable approach for surveillance of women treated for high-grade CIN. When considering co-testing, combining p16/Ki-67 with hrHPV testing leads to a sensitivity comparable to cytology/hrHPV co-testing, but a significantly higher specificity, resulting in higher PPV's and less referrals for colposcopy. Therefore, when considered in combination with hrHPV, p16/Ki-67 might be an attractive approach for follow-up of women treated for high-grade CIN and warrants further clinical performance evaluation studies with longer follow-up time.

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