HPV Cotesting of Unsatisfactory Papanicolaou Tests

Implications for Follow-up Intervals

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ABSTRACT

Objectives: The 2019 American Society of Colposcopy and Cervical Pathology management guidelines recommend that patients with an unsatisfactory Papanicolaou (Pap) test (UPT) and negative human papillomavirus (HPV) cotest undergo repeat age-based screening in 2 to 4 months. The rationale is that a negative HPV test in the setting of an UPT may reflect an inadequate sample and therefore should not be interpreted as truly "negative." For patients 25 years and older who are cotested, if HPV is positive for the 16 or 18 genotypes, direct referral for colposcopy is recommended. Our study aimed to determine if a negative HPV cotest result is predictive of the absence of a high-grade squamous intraepithelial lesion (HSIL) and whether these patients may be called back for repeat testing at an interval longer than 2 to 4 months.

Methods: Follow-up cervical cytology and biopsy results in women with UPT and HPV cotests from January 2017 to December 2021 were collected. Original UPT and HPV cotest results were correlated with the follow-up Pap and biopsy results.

Results: There were 1,496 (2.28%) UPT cases out of 65,641 total Pap tests. Among the 1,496 UPT cases, 1,010 (67.5%) had HPV cotesting; 676 (45.1%) were followed by repeat Pap or biopsy within 4 months and 850 (56.8%) within 12 months. The total follow-up rate was 81%, with a range of 3 days to 36 months. The HSIL rate in HPV-positive cases was 5.7% (3/53) vs 0.4% (2/539) ($P = .006$) in HPV-negative cases. In UPT, HPV cotesting showed negative predictive values for low-grade and high-grade squamous intraepithelial lesion detection of 98.5% and 99.6%, respectively, while positive predictive values were 19% and 5.7%.

Conclusions: A negative HPV cotest in individuals with UPT predicted the lack of HSIL in our study. Compliance with the recommended follow-up time of 2 to 4 months for women with UPT was low (45.1%). Our study suggests that women with UPT and negative HPV cotest may be safely called back at an interval longer than 4 months.

INTRODUCTION

Cervical cancer screening using the Papanicolaou (Pap) test has been immensely successful, with a substantial decrease in cervical cancer incidence and mortality rates in those countries in which screening was implemented.[1] Terminology for reporting cervicovaginal cytology was initially standardized by The Bethesda System (TBS) in 1988, with its newest revised version in 2014 (TBS 2014). Evaluation of specimen adequacy is considered by

KEY POINTS

- Follow-up cervical cytology and biopsy results in women with unsatisfactory Papanicolaou test (UPT) and human papillomavirus (HPV) cotests from January 2017 to December 2021 were collected. Original UPT and HPV cotest results were correlated.
- Our study provided sizable institutional data on UPT with HPV cotesting and follow-up data, highlighting the high negative predictive value of HPV for high-grade squamous intraepithelial lesion (99%).
- Our study suggests that women with UPT and negative HPV cotest may be safely called back at an interval longer than 4 months.

KEY WORDS

Unsatisfactory Pap test (UPT); HPV cotest; Follow-up interval; Squamous intraepithelial lesion; Cervical intraepithelial neoplasia; Cervical cancer; Screening

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many to be the single most critical quality assurance component of Pap tests. TBS 2014 provides feedback about specimen adequacy using the "satisfactory" or "unsatisfactory" categories: an unsatisfactory Pap test either shows scant squamous cellularity or has more than 75% of cells obscured in a specimen without any detectable abnormal features.[2] These Pap tests are considered unreliable for the evaluation of squamous lesions. Older studies based on conventional Pap smears have reported high unsatisfactory rates prior to diagnosis of the high-grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma in previously screened women.[2-9] The unsatisfactory rate has been reduced significantly by introducing liquid-based cytology (LBC) compared with conventional smears, with the increased recovery of squamous cells and a decrease in obscuring elements.[10] In addition, computer-imaged (CI) screening of LBC has been reported to be superior in detecting rare abnormal cells in samples that do not have adequate cellularity,[11] attesting to the increased sensitivity of this screening technology over conventional Pap smears. The detection rate of human papillomavirus (HPV) has also been shown to be very low in negative image screened LBC specimens.[12-16]

Despite newer studies showing the benefits of CI-screened LBC and HPV cotesting, a major issue related to an unsatisfactory Pap test (UPT) remains the potential for the sample not being representative and thus missing a high-grade lesion, necessitating patients to return for repeat testing in a short interval.[17] The 2019 American Society of Colposcopy and Cervical Pathology (ASCCP) management guidelines continue to recommend that individuals with an UPT and negative HPV cotest undergo repeat age-based screening in 2 to 4 months.[18,19] The rationale is that a negative HPV test in the setting of UPT may reflect an inadequate sample and therefore should not be interpreted as truly negative. Only a few studies have examined adherence to recommended guidelines for follow-up and outcome after an UPT with CI-screened LBC and the role of HPV testing in this setting.[12,17] Implementation of the CI screening significantly increased the detection of atypical squamous cells, cannot rule out HSIL (ASC-H); atypical glandular cells (AGCs); low-grade squamous intraepithelial lesion (LSIL); and HSIL but had no significant impact on the atypical squamous cells of undetermined significance (ASCUS) detection rate; meanwhile, the proportion of negative for intraepithelial lesion or malignancy (NILM) and unsatisfactory cases decreased significantly.[13-15] Our study aims to determine the usefulness of HPV cotesting in patients with CI-screened UPT for the detection of squamous intraepithelial lesion (SIL) and the follow-up adherence to the current guidelines after an UPT diagnosis.

MATERIALS AND METHODS

This is a study approved by the institutional review board. A retrospective cohort of patients with UPT diagnosed at our institution from January 2017 to December 2021 was tabulated. Available HPV cotesting, histologic follow-up, and subsequent Pap tests were retrieved from the institutional laboratory information system. Clinical data were extracted from electronic health records.

All cervicovaginal cytology reports were classified according to TBS 2014. All Pap tests were processed using the ThinPrep 2000 Processor according to the manufacturer's specifications (Hologic). Computer-assisted screening of ThinPrep slides was done using the ThinPrep Imaging System (TIS; Hologic).

Human papillomavirus testing was performed using the Roche cobas 4800 HPV Test System from samples collected in Cytyc Preservcyt Solution (ThinPrep). The cobas DNA-based HPV test is a highly automated assay for the detection of high-risk HPV (hrHPV) DNA in LBC specimens using real-time polymerase chain reaction (PCR) technology with a set of 16 PCR primers (8 forward and 8 reverse) that amplify a ~200-bp fragment of the L1 gene from all 14 hrHPV genotypes. HPV types are classified as 16, 18, or "other" (non-16/18 types, including HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).

Sensitivity, specificity, and positive and negative predictive values of HPV cotesting of UPT were calculated to determine the accuracy of LSIL and HSIL detection using follow-up histopathology as the gold standard method. In cases in which histopathology was not available, subsequent follow-up Pap test results were used as the gold standard. The atypical category included cases with follow-up Pap tests with ASCUS, ASC-H, or AGC without histologic follow-up. A time-to-event analysis with Kaplan-Meier curves and log-rank test was performed to evaluate the time to occurrence of SIL and HSIL. Fisher exact test was used to analyze categorical variables. The variables were considered statistically significant when the P value was less than .05.

RESULTS

Among 65,641 Pap smears in the study period, 1,496 (2.3%) were UPTs. The median age was 45 years (range, 20-99). Of the patients with available last menstrual period (LMP) date, 49% (531/1,080) were postmenopausal, and 4.7% (51/1,080) were pregnant or postpartum. In patients of reproductive age with available LMP date, 48% (364/757) had their Pap test collected within 14 days of LMP (246 within 7 days; 965 were of reproductive age, and 208 had no information on LMP). The demographic information of this cohort is summarized in **TABLE 1**. The total follow-up rate was 57% (850 patients), ranging from 3 days to 3 years. In total, 675 (45%) cases had a follow-up Pap test or histopathology within 4 months, while 806 (54%) had a follow-up within 12 months.

In total, 1,010 (67.5%) cases had HPV cotesting available. HPV was positive in 6.4% of cases (n = 65; 7/65 HPV 16, 2/65 HPV 18, and 56/65 HPV other) **TABLE 2**. A total of 592 Pap tests had available HPV cotesting and follow-up. **TABLE 3** summarizes the follow-up histopathology or Pap test information related to HPV status. UPT with positive HPV cotesting had significantly higher rates of subsequent HSIL (5.7% vs 0.4%; $P = .006$) and LSIL (19% vs 1.5%; $P < .0001$) and lower rates of NILM (68% vs 86%; $P = .001$) compared with HPV-negative Pap tests. Time-to-event analysis showed that the occurrence of HSIL ($P = .0063$) and SIL ($P < .0001$) **FIGURE 1** in HPV-positive cases was significantly earlier than in HPV-negative cases.

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TABLE 1 Clinical and Demographic Information of Patients With Unsatisfactory Pap Tests in This Cohort[a]

Characteristic	Value
Age, median (range), y	45 (20-99)
Postmenopausal[b]	531/1,080 (49.0)
Pregnant or postpartum[b]	51/1,080 (4.7)
Testosterone use[b]	7/1,080 (0.6)
Time from LMP and UPT (patients of reproductive age)[c]	
<7 days	246/757 (32.0)
<14 days	364/757 (48.0)
Pap test indication[d]	
Screening	1,067/1,323 (81.0)
Diagnostic	256/1,323 (19.0)

LMP, last menstrual period; UPT, unsatisfactory Papanicolaou test.
[a]Values are presented as number (%) unless otherwise indicated.
[b]Not available in 416 cases.
[c]Not available in 208 cases.
[d]Not available in 173 cases.

TABLE 2 Follow-up of Unsatisfactory Papanicolaou Test Cases With HPV Cotesting Results

HPV status	Cases with follow-up, No. (%)	Cases without follow-up, No. (%)	*P* Value
HPV positive	53 (81.0)	12 (19.0)	<.0001
HPV 16	7	0	
HPV 18	1	1	
HPV non-16/18	45	11	
HPV negative	539 (57.0)	406 (43.0)	
Total	592 (59.0)	418 (31.0)	

HPV, human papillomavirus.

Sensitivity, specificity, and positive and negative predictive values for LSIL detection of HPV cotesting in UPT were 56%, 92.5%, 19%, and 98.5%, respectively. Sensitivity, specificity, and positive and negative predictive values for HSIL detection were 60%, 91.4%, 5.7%, and 99.6%, respectively **TABLE 4**.

On follow-up, 5 cases of high-grade SIL were identified; of those, 3 were HPV positive in the initial UPT (1 with HPV 16 and 2 with HPV other). The other 2 patients were initially HPV negative on cotesting; however, the subsequent HPV testing performed 12 days and 30 days after the initial cotesting showed positivity for HPV other and HPV 16, respectively. Most cases with follow-up HPV testing maintained the same HPV status (92% of HPV-positive and 96% of HPV-negative cases), while only 4% of HPV-negative cases converted to HPV positive with a median time between HPV tests of 34 days **TABLE 5**.

There were 18 cases of LSIL on follow-up. Ten of these were HPV positive on initial cotesting (5 HPV 16, 1 HPV 18, and 4 HPV other), while 8 were HPV negative. Six of 8 cases with initial HPV-negative cotesting had subsequent HPV testing and showed positivity in 4 (1 HPV 16 and 3 HPV other) cases with an interval between the first HPV test and follow-up ranging from 18 days to 1 year.

DISCUSSION

The Bethesda System for reporting cervicovaginal cytology results provides feedback on specimen adequacy using the "satisfactory" or "unsatisfactory" categories, and it defines UPT when the squamous cell count is less than 5,000 in liquid-based preparations (or fewer than 8,000 cells in conventional smear preparations) or when more than 75% of the squamous cell component is poorly visualized and/or obscured in the absence of cytomorphologic abnormalities. The 2019 ASCCP management guidelines recommend that women with UPT and negative HPV cotest results should undergo repeat age-based screening in 2 to 4 months.[18] For patients 25 years and older who are cotested without genotyping, if the HPV test is positive, repeat cytology in 2 to 4 months or colposcopy is acceptable. If HPV 16 or 18 is positive by genotyping, a direct referral for colposcopy is recommended.

The rationale for calling back patients for repeat testing in 2 to 4 months in the UPT/HPV-negative population is that a negative HPV test in the setting of UPT may reflect an inadequate sample due to insufficient squamous cellularity and therefore may not be truly negative.[18] However, HPV testing in the setting of unsatisfactory cytology specimens has not been well studied.[20] There are many factors associated with the adequacy of HPV tests in this setting, such as the type of HPV platform used and the reason for unsatisfactory Pap test results (the number of squamous cells, presence/absence of transformation zone elements, and presence of obscuring elements that makes the squamous component poorly visualized to the cytopathologist). Our institution uses the cobas 4800 system, which has an internal control for DNA amplification (β-globin); the internal control is a quality measure that indicates if there are enough cells for the validity of a negative HPV test. The cases that would raise concern for insufficient squamous cellularity for reliable HPV test results are those with a negative internal control; in this situation, the HPV test is flagged and reported as "invalid."[21] The argument for a falsely negative HPV test would be that the DNA amplified in an unsatisfactory Pap test could potentially come from cells other than the cervical squamous cells, such as inflammatory cells in Pap tests that contain significant inflammation and/or blood. In these cases, a correlation with the Pap result and an explanatory note if the HPV test is negative would be beneficial. In addition, the number of HPV-infected cells needed for HPV detection on the cobas platform depends on the HPV subtype. For example, HPV 16 and HPV 18 only need 600 cells/mL, while HPV 52 needs 2,400 cells/mL.[22] Regardless of the HPV subtype, these numbers are much lower than the 5,000 cell adequacy criteria for Pap tests.

In our study, the institutional unsatisfactory rate was 2.3%. This rate falls between the median and the 75th percentile of the surveyed laboratories, according to the College of American Pathologists ThinPrep reporting data.[23] The most common reason for UPT was postmenopausal status, followed by sampling close to or during the menstrual period (within 14 days of the menstrual cycle). These findings are similar to other published reports.[24] Only 45% of women had a follow-up within the recommended time frame of 2 to 4 months in our study. This low rate may be due to multiple factors, including but not limited to women's reluctance and anxiety to undergo repeat pelvic examination due to its uncomfortable nature

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TABLE 3 Histologic or Cytologic Follow-up of Unsatisfactory Pap Tests According to HPV Results

HPV status	Follow-up (Pap test and/or histology), No.			LSIL, No.	HSIL, No.	Total No.
	Unsatisfactory	NILM/benign	Atypical			
HPV positive	0	36	4	10	3	53
HPV negative	36	466	27	8	2	539
Total	36	502	31	18	5	592

HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy; Pap, Papanicolaou.

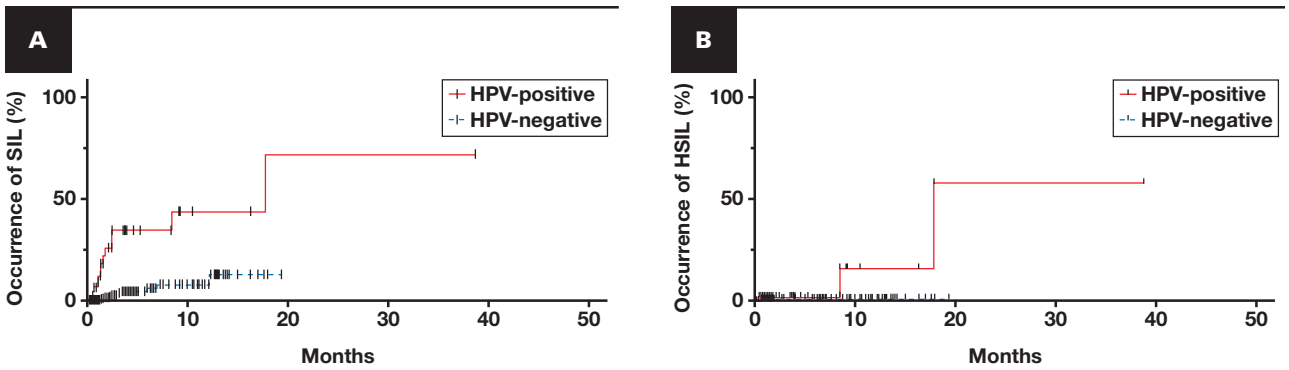

FIGURE 1 Time-to-event analysis of unsatisfactory Papanicolaou test for the occurrence of squamous intraepithelial lesion (SIL) ($P < .0001$) (**A**) and high-grade squamous intraepithelial lesion (HSIL) ($P = .0063$) (**B**) according to human papillomavirus status.

TABLE 4 Sensitivity, Specificity, and Positive and Negative Predictive Values of Human Papillomavirus Cotesting in Unsatisfactory Papanicolaou Tests in the Detection of LSIL and HSIL

Detection	Sensitivity, %	Specificity, %	PPV, %	NPV, %
LSIL detection	56.0	92.5	19.0	98.5
HSIL detection	60.0	91.4	5.7	99.6

HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NPV, negative predictive value; PPV, positive predictive value.

TABLE 5 Unsatisfactory Papanicolaou Tests With Initial and Follow-up HPV Test Results

Initial HPV status	Cases with follow-up HPV, No./total No. (%)	HPV positive on follow-up, No./total No. (%)	Interval between initial HPV and positive HPV follow-up, d (range)
HPV positive	26/65 (40.0)	24/26 (92.0)	29 (8-365)
HPV negative	415/945 (44.0)	17/415 (4.0)	34 (9-407)

HPV, human papillomavirus.

and the inconvenience of scheduling another doctor's visit. The follow-up rate was much higher in the HPV-positive group (81%) compared to the HPV-negative group (57%) ($P < .05$) **TABLE 2**, indicating better adherence to management guidelines when HPV is positive.

All Pap tests in our institution are screened using computer-assisted technology (TIS). The TIS has a lower unsatisfactory rate and a similar or increased sensitivity compared with manual screening, with significantly increased detection rates of high-grade SIL, ranging from 24% to 42%.[15,25-27] A study similar to ours using TIS but the Hybrid Capture 2 method for HPV testing showed that a positive HPV result in women with UPT effectively identified those at risk for SIL on follow-up. Negative HPV results had a high negative predictive value. The overall risk for SIL on follow-up of all patients with TIS and UPT was lower compared with women who underwent manual screening with conventional Pap smears.[12]

In our study, a negative HPV cotest in women with UPT was highly indicative of a lack of a precursor lesion with a negative predictive value of 98.5% and 99.6% for LSIL and HSIL, respectively. Furthermore, 32% of women with UPT and positive HPV cotest result had atypical (7.5%), LSIL (19%), or HSIL (5.5%) on follow-up **TABLE 3**. Even with a longer follow-up time of up to 12 months, there were only 2 (0.4%) HSIL cases in the HPV-negative group out of 539 patients, which seemed to represent an initial false-negative HPV test since a repeat HPV test was positive within 12 and 30 days. A false-negative HPV test in UPT may be due to several reasons in addition to insufficient cellularity, such as cervical parakeratosis/hyperkeratosis,[28] older age group (>50 years),[29] and lesions either in early stages or in regressing stages.[30] Considering that a lesional cervix will have a lower rate of UPT,[31] and our results showed an exceedingly low HSIL rate among the UPT/negative HPV group, we suggest that patients with UPT and negative HPV cotest may be safely called back at an interval of longer than 2 to 4 months. These findings are similar to the previous study by Zhao and Austin,[12] which showed a high negative predictive value of HPV testing in the UPT setting with an interval follow-up of 5.9 months. The time-to-event analysis

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of an unsatisfactory Pap test for the occurrence of SIL FIGURE 1A and HSIL FIGURE 1B according to HPV status also demonstrates that within 20 months of follow-up comparison, the HPV-negative cotest patient group shows statistically significant differences compared with the HPV-positive cotest UPT group for developing SIL and HSIL, especially the development of HSIL rate, which is exceedingly low ($P < .01$).

One of our study's limitations is its retrospective nature; therefore, we were not able to obtain histologic follow-up in a percentage of cases. Of particular interest, a portion of cases with an atypical cytology Pap test was not followed up histologically (n = 31); therefore, these cases were not included in the final analysis due to a lack of definitive tissue diagnosis. The reasons for excluding these patients from analysis include the relatively small number of patients in this group, the majority of atypical cases being HPV negative (87%), and most cases with histologic follow-up being benign on biopsy (76%). We believe that the inclusion of these cases would add minimal additional data and possibly introduce bias in the final analysis. Additional studies with longer follow-up would be helpful in clarifying management recommendations for this particular group.

It is very important to consider the testing platform when evaluating any HPV test as a screening modality, given that there are different sensitivities and specificities to the various platforms in clinical use. Several methods/platforms exist to detect HPV in Pap test specimens, such as DNA, messenger RNA (mRNA), and in situ hybridization. While the HPV mRNA test has higher specificity than the other 2 assays, which can reduce referral to colposcopy, HPV DNA tests have the highest sensitivity and are more suitable for a screening test.[7] The HPV test method we are using at our institution is the Roche cobas 4800 HPV Test System, a highly automated DNA-based HPV test approved by the US Food and Drug Administration that has relatively high sensitivity in the clinical setting, with an absolute sensitivity of 90% to 98.3% and a relative sensitivity of 0.98 to 1.[32-35] This test is one of the assays fully matching the current criteria based on reproducibility and noninferior accuracy defined by Meijer et al[36] and thus can be recommended in HPV-based cervical cancer screening using clinician-collected cervical samples.[32] In fact, HPV testing alone has proven to be more sensitive for detecting HSIL than cytology.[8] In addition, it has been shown that for general screening of populations, a negative HPV test is more accurate in reassuring absence of a high-grade lesion in comparison to cytology, which has a higher false-negative rate.[37-43] Our data confirmed that a negative hrHPV result associated with UPT has a high negative predictive value.

Meanwhile, it is essential to understand the reasons for UPT and find solutions to minimize the UPT rate. Liquid-based cytology alleviates most causes of UPT, such as obscuring blood, poor fixation, cytolysis, and uneven distribution and transfer of collected cells. Scant cellularity remains the primary cause of unsatisfactory LBC.[10] The presence of red blood cells from hemorrhagic collections, mucus, and proteinaceous debris can clog the ThinPrep device's filter, which leads to insufficient cellularity.[44] Reprocessing the remaining sample with glacial acetic acid has shown to improve the UPT rate.[45]

Older age is associated with increased rates of UPT.[46] The UPT rate is exceptionally high in peri- and postmenopausal patients,[47] constituting nearly half of this cohort. In our study, a significant proportion of UPT in patients of reproductive age had the sample collected within 7 and 14 days of the LMP. Sampling the cervix during the menstrual period is one of the major factors associated with UPT in our cohort. Another factor in the younger population that can affect the UPT rate is the use of testosterone in transgender patients, which leads to an atrophic effect on the squamous epithelium of the cervix.[48,49]

In summary, our study provided sizable institutional data on UPT with HPV cotesting and follow-up data, highlighting the high negative predictive value of HPV for detecting HSIL (99%) TABLE 5. In addition, these results provide evidence for the feasibility and safety of extending the follow-up period beyond 2 to 4 months in patients with UPT and negative HPV cotesting.

Conflict of interest disclosure: The authors have nothing to disclose.

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