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Original Article

Triage performance of DNA methylation for women with high-risk human papillomavirus infection

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Abstract

Objective: DNA methylation is a promising biomarker for cervical cancer screening. This study aimed to validate the triage performance of cytological DNA methylation for detecting cervical intraepithelial neoplasia of grade 3 or worse (CIN3+) in women with high-risk human papillomavirus (hrHPV) infection from a large prospective cohort undergoing opportunistic screening in China (METHY3).

Methods: The triage performance for detecting CIN3+ lesions was compared between HPV16/18 genotyping, a liquid-based cytology (LBC) test, and the *PAX1* and *JAM3* methylation (*PAX1*^{m}/*JAM3*^{m}) test according to cervical pathologic outcomes. Among the 4394 women infected with hrHPV, 1105 had definitive cervical histological findings that were analyzed.

Results: For detecting CIN3+, the specificity of HPV16/18(+), the LBC result of ≥atypical squamous cells of undetermined significance (ASCUS), and *PAX1*^{m}/*JAM3*^{m}(+) was 66.4%, 23.9%, and 89.6%, respectively, with odds ratios of 4.24 (95% confidence interval [CI], 2.85-6.40), 4.44 (2.27-10.1), and 18.5 (12.1-28.7) ($P < .001$), respectively. *PAX1*^{m}/*JAM3*^{m}(+) had the highest area under the receiver operating characteristic curve (0.790, 95% CI, 0.747-0.832) in the whole cohort and in women of various ages. *PAX1*^{m}/*JAM3*^{m} (+) was detected in all patients with cancer ($n = 28$). Compared with HPV16/18 genotyping and the LBC test, *PAX1*^{m}/*JAM3*^{m} testing reduced referrals to colposcopy by 20.64 percentage points and 61.18 percentage points, respectively.

Conclusions: *PAX1*^{m}/*JAM3*^{m} testing is highly specific for detecting CIN3+. As a triage biomarker, it is superior to HPV 16/18 genotyping and LBC testing for women with hrHPV infection.

Keywords: cervical screening; DNA methylation; CIN3; HPV genotype; cytology test.

Implications for practice

The gene of PAX1/JAM3 methylation test significantly increased the specificity of screening CIN3+ in women with minimally abnormal cytological results, especially in younger women.

Introduction

Primary human papillomavirus (HPV) testing is increasingly regarded as the preferred approach for cervical cancer screening.[1,2] HPV-based cervical screening is highly sensitive and objective for detecting high-grade cervical intraepithelial neoplasia (CIN) and cervical cancer, but its specificity is suboptimal.[3] Most women infected with HPV experience a transient infection that resolves naturally[4]; therefore, many colposcopy referrals and associated cervical excisional treatments are unnecessary and could be reduced with better triage tests. The use of a liquid-based cytology (LBC) test and/or HPV 16/18 genotyping is recommended as an additional triage test to identify women who require further investigation.[5] Unfortunately, these methods are not specific enough,[6] and in many settings, trained cytologists are in short and ever-dwindling supply.[7] The ability to classify hrHPV-infected women with increased accuracy, reliability, and reproducibility remains an important challenge.

DNA methylation of tumor suppressor genes in promoter regions is a hallmark of progressive oncogenesis.[7,8] The methylation levels of specific tumor suppressor genes in the cervix increase with the severity of CIN, peaking in patients with

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cervical cancer.[9-11] DNA methylation testing has become a promising cervical screening method owing to its good performance and reproducibility.[12-14] In addition, DNA methylation can be used as a triage tool in women with high-risk HPV (hrHPV) infection.[15-17] *PAX1* and *JAM3* have shown great potential as methylated targeted host genes for cervical cancer screening.[18-21] However, the effects of these methods on triage outcomes have not been studied in depth.

In a multicenter prospective study (METHY3, NCT04646954), more than 20,000 women who underwent opportunistic screening were subjected to LBC, hrHPV, and *PAX1/JAM3* methylation (*PAX1^m/JAM3^m*) testing. As part of the METHY3 study, we selected patients with hrHPV from Peking Union Medical College Hospital (PUMCH) and evaluated the triage performance of an LBC outcome of ≥atypical squamous cells of undetermined significance (ASCUS), the HPV16/18 genotype, and the *PAX1^m/JAM3^m* test result for detecting cervical intraepithelial neoplasia grade 3 or worse (CIN3+).

Materials and methods

Participants and study design

This study included eligible women who underwent opportunistic cervical cancer screening, which included hrHPV genotyping, LBC testing, and *PAX1^m/JAM3^m* testing, between November 2020 and December 2021 at PUMCH. The triage performance for detecting CIN3+ in women with hrHPV infected was compared between LBC ≥ ASCUS, HPV16/18 genotyping, *PAX1^m/JAM3^m* testing, and their combinations according to cervical histology achieved by colposcopy or other surgical procedures.

The inclusion criteria were as follows: 18 years of age or older; intact cervix; no serious immunodeficiency conditions of HIV infection; history of organ transplantation; treatment with immunosuppressive drugs; and full consent. Conversely, we excluded women who had known malignant tumors of the female genital tract or who had active malignant tumors in other sites that were still under treatment. All participants provided informed consent.

Sample collection, cytology testing, and hrHPV genotyping

All eligible participants underwent gynecological examinations and cervical cytology sampling. The cytology samples were sent for hrHPV and LBC assays, and the residual samples were sent for *PAX1/JAM3* methylation assays.

All the samples were subjected to high-performance HPV testing on a Cobas 4800 system (Roche Molecular Systems, Inc.) according to the manufacturer's instructions.[22] The Cobas HPV test is an in vitro quantitative technique for detecting high-risk HPV DNA via polymerase chain reaction amplification that can detect HPV16, HPV18, and other HPV12 types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).

The LBC and imaging system manual were used for cytology tests (Hologic, Inc.). Cytologists used the 2014 Bethesda system[23] to classify the cytology results.

DNA methylation assays

Methylation was detected in a certified DNA laboratory, and the operators and staff members were blinded to the patients' clinical information, LBC test results, HPV genotyping results, and cervical histopathology results. Genomic DNA (gDNA) was extracted from the exfoliated cervical sample by using the JH-DNA Isolation and Purification Kit (OriginPoly Bio-Tec Co., Ltd.) according to the manufacturer's instructions. Moreover, the DNA concentration was quantified via a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). The *PAX1^m* and *JAM3^m* levels were determined via the Human PAX1 and JAM3 Gene Methylation Detection Kit (real-time PCR) for Cervical Cancer [Class III medical devices approved by the National Medical Products Administration (No. 20233400253)] with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (OriginPoly Bio-Tec Co., Ltd.). The hypermethylation level of the *PAX1* and *JAM3* genes was determined by the difference between the two Ct values (ΔCt *PAX1* = Ct *PAX1*-Ct *GAPDH* and ΔCt *JAM3* = Ct *JAM3*-Ct *GAPDH*). In accordance with the manufacturer's instructions, a positive CISCER (*PAX1^m/JAM3^m*) test result was defined as ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0.

Histology evaluation

Cervical histology was obtained by colposcopy examination, which was performed according to the current guidelines,[24,25] or by other surgical procedures, such as hysterectomy, for indications other than cervical lesions. Two pathologists independently reviewed the histological results. The *PAX1^m/JAM3^m* results were not indicated for colposcopy referrals or cervical biopsies in this study.

Statistical analysis

SPSS 26.0 (IBM Corp.=) and R (version 4.1.2) were used for all the statistical analyses. The participants were characterized using descriptive statistics. The quantitative data are represented as $x^2 \pm s$. The ΔCt values of methylated *PAX1* and *JAM3* were compared between groups via one-way ANOVA. The measurement data are shown as %, while comparisons between groups were analyzed by the chi-square test. Receiver operating characteristic (ROC) curves were drawn to evaluate the areas under the curve (AUC) for CIN3+ detection. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value for detecting CIN3+ lesions were calculated with 95% confidence intervals (CIs). The sensitivity and specificity for detecting CIN3+ lesions were compared via McNemar's test. All tests were two-sided, and differences were considered statistically significant at $P < .05$ for 95% CIs.

Results

Patient clinicopathological characteristics

Among the 20 456 women included in the METHY3 study at the PUMCH, 4394 were identified as being infected with hrHPV (Figure 1). Table 1 summarizes the cohort's demographic characteristics and clinical information.

In total, 1105 women had cervical histology results: 615 (55.7%) with normal findings or inflammation, 255 (23.1%) with CIN1, 112 (10.1%) with CIN2, 95 (8.6%) with CIN3, and 28 (2.5%) with cervical cancer. The median age was 42 (interquartile range: 34–53) years, with 115 (10.4%), 625 (56.5%), and 365 (33.0%) women aged younger than 30 years, 30–49 years, and older than 50 years, respectively. LBC ≥ ASCUS, HPV16/18 (+), and *PAX1^m/JAM3^m* (+)

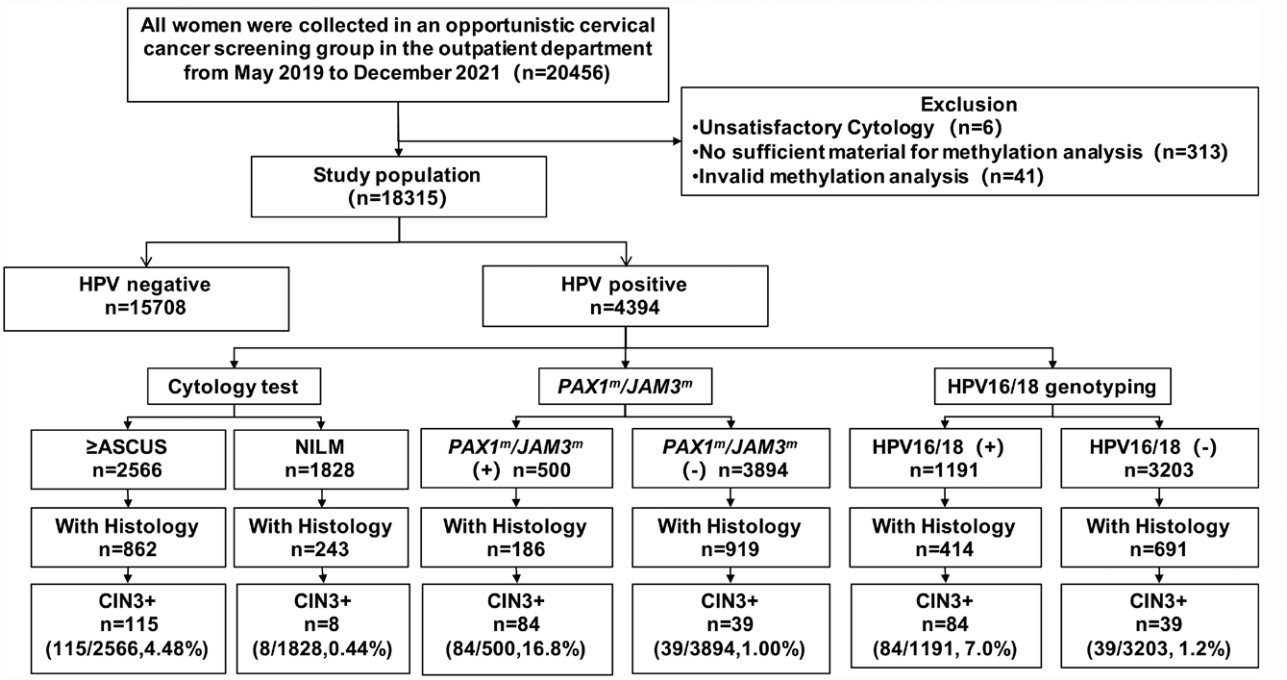

Figure 1. Flowchart of the cervical sample collection cohort. Abbreviations: ASCUS, atypical squamous cells of undetermined significance; CIN3+, cervical intraepithelial neoplasia grade 3 or worse; HPV-positive, infection with one or more strains of HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 or 68; NILM, no intraepithelial lesions or malignancy.

Table 1. Characteristics of the participants.

Characteristic	Overall	Unknown	Normal	CIN1	CIN2	CIN3	Cervical cancer	*P* value
n.	4394	3289	615	255	112	95	28	
AGE (median (IQR))	42(34-53)	43 (34-54)	43(35-56)	39(32-51)	38(31-48)	41(35-52)	51(42-64)	<.001
HPV Genotyping (%)								
HPV16/18	1191 (27.1)	777 (23.6)	194 (31.5)	81 (31.8)	55 (49.1)	59 (62.1)	25 (89.3)	<.001
HPV others	3203 (72.9)	2512 (76.4)	421 (68.5)	174 (68.2)	57 (50.9)	36 (37.9)	3 (10.7)	
LBC (%)								
NILM	1828 (41.6)	1585 (48.2)	183 (29.8)	38 (14.9)	14 (12.5)	5 (5.3)	3 (10.7)	<.001
≥ASCUS	2566 (58.4)	1704 (51.8)	432 (70.2)	217 (85.1)	98 (87.5)	90 (94.7)	25 (89.3)	
PAX1^{m}(+) (%)	418 (9.5)	258 (7.8)	36 (5.9)	18 (7.1)	26 (23.2)	53 (55.8)	27 (96.4)	<.001
JAM3^{m} (+) (%)	313 (7.1)	179 (5.4)	28 (4.6)	14 (5.5)	20 (17.9)	46 (48.4)	26 (92.9)	<.001
PAX1^{m}/*JAM3*^{m} (+) (%)	500 (11.4)	314 (9.5)	52 (8.5)	21 (8.2)	29 (25.9)	56 (58.9)	28 (100.0)	<0.001

Abbreviations: ≥ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; hrHPV, high-risk human papillomavirus; IQR, interquartile range; *JAM3*^{m}(+), ΔCt *JAM3* ≤ 10.0; NILM, no intraepithelial lesions or malignancy; *PAX1*^{m}(+), ΔCt *PAX1;* ΔCt*PAX1*^{m}/*JAM3*^{m}(+), ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0.

accounted for 78.0% (862/1105), 37.5% (414/1105), and 16.8% (186/1105) of the whole cohort, respectively.

Relationship of screening results with cervical histology

Figure 2A-D shows the proportions of patients with HPV16/18(+), LBC ≥ ASCUS, and *PAX1*^{m}/*JAM3*^{m}(+) results within each histological subgroup. The rate of LBC ≥ ASCUS results was generally high across all groups, including the normal and CIN1 groups. As the cervical lesion grade increased, the proportions of HPV16/18(+), *PAX1*^{m}(+), *JAM3*^{m}(+), and *PAX1*^{m}/*JAM3*^{m}(+) results also increased. *PAX1*^{m}/*JAM3*^{m} (+) results was detected in all cancer patients (*n* = 28).

Performance of various screening methods and their combinations

The AUC of *PAX1*^{m}/*JAM3*^{m} (+) for detecting CIN3+ was 0.790 (95% CI, 0.747-0.832) for all women and 0.669 (0.489-0.849), 0.777 (0.720-0.833), and 0.829 (0.764-0.893) for women aged less than 30, 30–49, and ≥50 years, respectively (Supplementary Material Figure S1 A-D).

Table 2 lists the diagnostic accuracy of the different screening methods and their combinations in the whole cohort, and Supplementary Material Table S1 lists the diagnostic accuracy of the different screening methods and their combinations in various age groups. In the whole cohort and all age groups, *PAX1*^{m}/*JAM3*^{m} testing showed the highest specificity

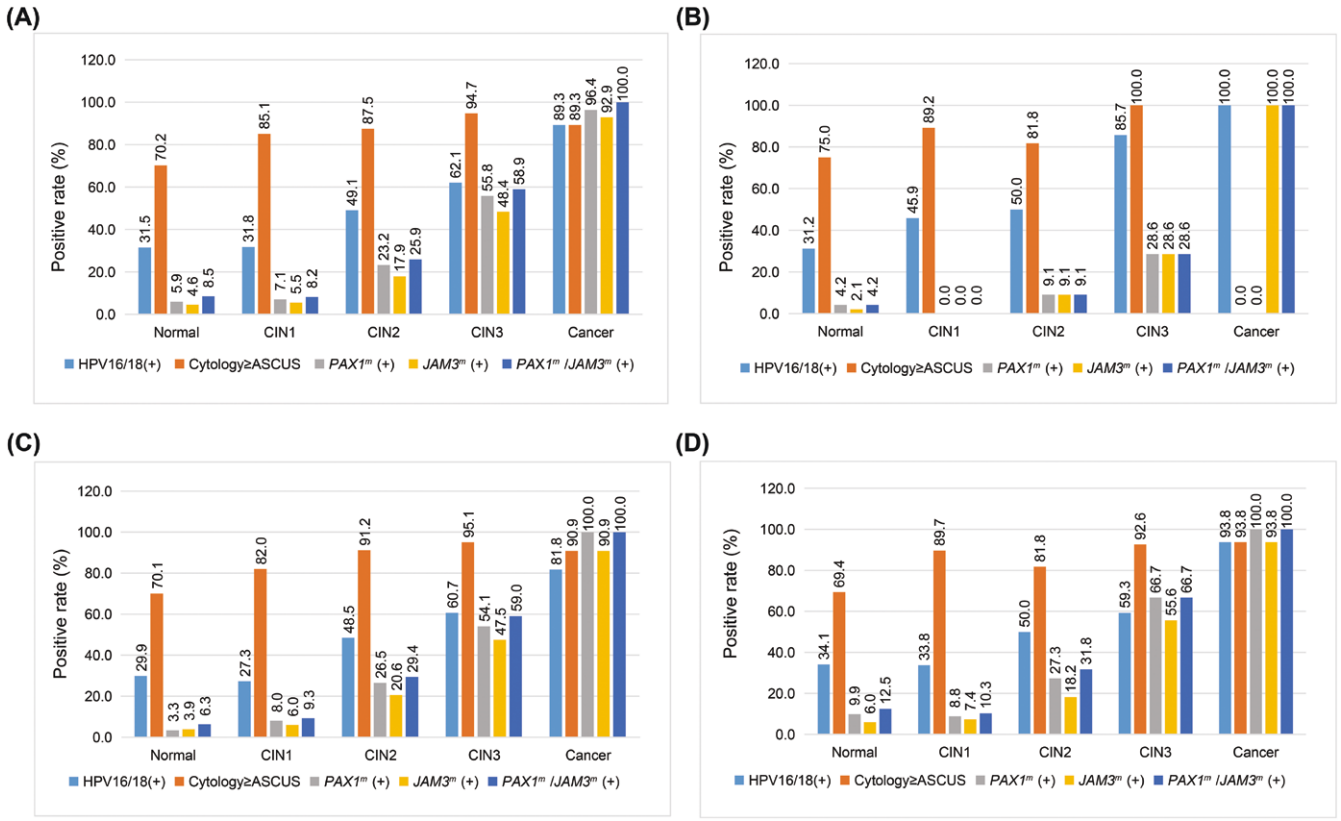

Figure 2. Positivity rates for HPV 16/18, LBC ≥ ASCUS, and *PAX1*^{m}/*JAM3*^{m} in the histological subgroups. (A) Numbers of total women. (B) Women younger than 30 years of age. (C) Women aged 30-49 years. (D) Women over 50 years of age. Abbreviation: ASCUS, atypical squamous cells of undetermined significance.

Table 2. Analysis of triage performance of human papillomavirus 16/18, liquid-based cytology, and *PAX1*^{m}/*JAM3*^{m} for detecting cervical intraepithelial neoplasia 3+ in women with high-risk human papillomavirus infection.

Target	AUC (95% CI)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)	Referral cases *n*, %
HPV 16/18 (+)	0.673(0.630-0.717)	68.3(60.1-76.5)	66.4(63.4-69.3)	20.3(16.4-24.2)	94.4(92.6-96.1)	414, 37.47%
LBC ≥ ASCUS	0.587(0.562-0.613)	93.5(89.1-97.9)	23.9(21.3-26.6)	13.3(11.1-15.6)	96.7(94.5-99.0)	862, 78.01%
PAX1^{m}/*JAM3*^{m} (+)	0.790(0.747-0.832)	68.3(60.1-76.5)	89.6(87.7-91.5)	45.2(38.0-52.3)	95.8(94.5-97.1)	186, 16.83%
HPV 16/18 (+) or LBC ≥ ASCUS	0.575(0.561-0.589)	99.2(97.6-100.0)	15.9(13.6-18.2)	12.9(10.7-15.0)	99.4(98.1-100.0)	948, 85.79%
HPV 16/18 (+) or *PAX1*^{m}/*JAM3*^{m} (+)	0.742(0.710-0.775)	88.6(83.0-94.2)	59.9(56.8-62.9)	21.7(18.1-25.3)	97.7(96.5-98.9)	503, 45.52%
LBC ≥ ASCUS or *PAX1*^{m}/*JAM3*^{m} (+)	0.601(0.582-0.620)	97.6(94.8-100.0)	22.7(20.1-25.3)	13.7(11.4-15.9)	98.7(97.2-100.0)	879, 79.55%

Abbreviations: AUC, area under curve; HPV 16/18(+), human papillomavirus 16 or 18 types positive; HPV 16/18(+) or LBC ≥ ASCUS, either HPV16/18 positive or ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0 called positive; HPV 16/18(+) or *PAX1*^{m}/*JAM3*^{m}(+), either HPV16/18 positive or liquid-based cytology results atypical squamous cells of undetermined significance or worse called positive; LBC ≥ ASCUS, liquid-based cytology results atypical squamous cells of undetermined significance or worse; LBC ≥ ASCUS or *PAX1*^{m}/*JAM3*^{m}(+), either liquid-based cytology results atypical squamous cells of undetermined significance or worse or ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0 called positive; NPV, negative predictive value; *PAX1*^{m}/*JAM3*^{m}(+): ΔCt PAX1 ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; PPV, positive predictive value.

and diagnostic AUC for CIN3+, leading to the fewest referrals to cervical biopsy. In the whole cohort, LBC ≥ ASCUS, HPV16/18(+), and *PAX1*^{m}/*JAM3*^{m}(+) results as triage methods led to referral rates of 78.01%, 37.47% and 16.83%, respectively. Figure 3A shows the current cervical cancer screening protocol for women with hrHPV infection. Figure 3B shows the combined hrHPV testing and *PAX1*^{m}/*JAM3*^{m} detection protocol used for cervical cancer screening.

Risk of CIN3+ suggested by HPV16/18(+), LBC ≥ ASCUS, and *PAX1*^{m}/*JAM3*^{m}(+) results

As shown in Table 3, *PAX1*^{m}/*JAM3*^{m} (+) results suggested a greater risk of developing CIN3+ (OR: 18.5; 95% CI, 12.1-28.7) than did HPV16/18(+) results (4.24, 2.85-6.40) or LBC ≥ ASCUS results (4.44, 2.27-10.1). HPV16/18(+) and LBC ≥ ASCUS results predicted a greater risk of developing CIN3 + ($P > .05$).

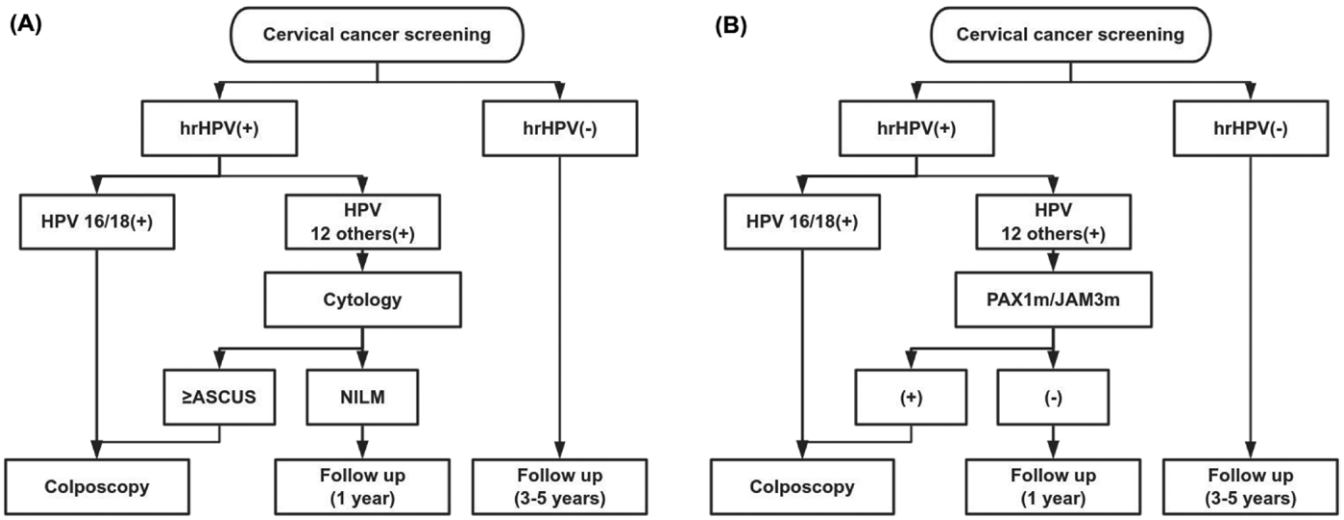

Figure 3. Cervical cancer screening protocol. (A) Current cervical cancer screening protocol. (B) Combined hrHPV testing and *PAX1*^{m}/*JAM3*^{m} detection as a cervical cancer screening protocol. Abbreviations: HPV 16/18+, human papillomavirus 16 or 18 types positive; HPV 12 other(+), infection with one or more strains of HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 or 68; hrHPV(+), high-risk human papillomavirus positive; NILM, no intraepithelial lesions or malignancy; *PAX1*^{m}/*JAM3*^{m}(+), ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0;≥ASCUS, Liquid-based cytology results atypical squamous cells of undetermined significance or worse.

Table 3. The odd risks of human papillomavirus genotypes tests, liquid-based cytology, and *PAX1*^{m}/*JAM3*^{m} for cervical intraepithelial neoplasia 3+.

	≤CIN2 (*n* = 982)	≥CIN3 (*n* = 123)	ORs (95%CI)	*P* values
hrHPV				<.001
HPV 12others +	652 (66.4%)	39 (31.7%)	Reference	
HPV16/18+	330 (33.6%)	84 (68.3%)	4.24 (2.85-6.40)	
LBC results				<.001
NILM	235 (23.9%)	8 (6.50%)	Reference	
≥ASCUS	747 (76.1%)	115 (93.5%)	4.44 (2.27-10.1)	
PAX1^{m}/*JAM3*^{m}				<.001
(-)	880 (89.6%)	39 (31.7%)	Reference	
(+)	102 (10.4%)	84 (68.3%)	18.5 (12.1-28.7)	

Abbreviations: ASCUS, atypical squamous cells of undetermined significance; NILM, no intraepithelial lesions or malignancy; ORs, odds ratio; *PAX1*^{m}/*JAM3*^{m}, ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; 95%CI, 95% Confidence Interval.

Discussion

This prospective study investigated the clinical utility of *PAX1*^{m}/*JAM3*^{m} detected as a triage test for women infected with hrHPV via routine cervical screening. The ΔCt values of *PAX1* and *JAM3* methylation increased as histopathological severity increased, as the median *PAX1* and *JAM3* gene methylation scores increased with all increases in histology levels from CIN2, CIN2, and CIN3 to cancer. Furthermore, this study comprehensively evaluated the triage capabilities of various detection methods for women with infected hrHPV who undergo cervical cancer screening. These results indicate that the use of *PAX1*^{m}/*JAM3*^{m} from cervical epithelial cells is highly accurate for detecting CIN3 and more severe CIN3+ lesions. In addition, *PAX1*^{m}/*JAM3*^{m}(+) was detected in 100% of cervical cancers. Therefore, testing methods involving host gene methylation can accurately identify high-risk cervical lesions and are promising for triaging cervical cancer.

Although primary HPV tests have improved cervical cancer screening in China, they also cause some problems, including overscreening, overtreatment, and a high psychological burden on patients.[26] HPV infection is detected in tens of millions of women via routine cervical screenings every year, causing them to experience panic and anxiety. Therefore, accurate triage of hrHPV infection screening samples is one of the most pressing scientific issues that must be addressed for the modernization of primary HPV-based screening for cervical cancer prevention. Cytological examination is still the preferred classification method, but it depends heavily on the subjective skills of cytologists. Only countries with high-quality cytology can conduct relatively balanced HPV screening in terms of screening tests and overreference. Moreover, repeated abnormal cytology and colposcopy referrals escalate cancer fear, disgust/shame, surprise, and sexual concerns.[27,28] The hrHPV DNA reportedly changes viral gene expression and the host chromosome structure and leads to epigenetic modifications when it is integrated into the host genome.[29,30] In particular, epigenetic alterations, such as DNA methylation and acetylation, histone modifications, and lncRNAs, affect gene expression and genomic stability and promote cancer development.[31,32] Similarly,

methylation testing has proven useful as an additional triage tool for women infected with hrHPV in primary screening in numerous studies abroad. The PROHTECT-3 study demonstrated that the use of *MAL* and *miR-124-2* methylation for the triage detection of CIN2+ in women infected with HPV is not inferior to cytology.[33] The combination of *FAM19A4/miR124-2* methylation and HPV16/18 can be used as a triage tool for detecting HPV positivity.[34] Moreover, *FAM19A4/miR-124-2* combined with HPV16/18 can also be used for CIN3+ detection.[35] *FAM19A4/miR124-2* methylation is equivalent to cytology for the triage of CIN3+ patients in HPV screening-based populations.[34] The methylation of four genes, namely, *JAM3*, *EPB41L3*, *C13ORF18*, and *TERT*, can be used in a triage protocol for women infected with HPV.[36] For women with hrHPV, S5 testing, which is based on the combination of HPV 16, 18, 31, and 33 DNA methylation and the human host gene *EPB41L3,* is superior to HPV16/18 for triage, either for all women or specifically for women with abnormal cytological findings.[15,37,38] In low- and middle-income countries, the S5 test is superior to HPV 16/18 or cytology for triage in women infected with hrHPV.[15] In this study, *PAX1^m/JAM3^m*(+) results demonstrated high specificity (89.6%) and a low colposcopy referral rate (16.83%), which was better than those of LBC ≥ ASCUS results (23.9% and 78.01%, respectively) and HPV 16/18(+) results (66.4% and 37.47%). Owing to the high specificity found in this study, we propose that methylation is a triage process that can be used as an alternative to cytology for women infected with hrHPV.

In this study, we demonstrated that a PCR-based DNA methylation signature—that is, the *PAX1^m/JAM3^m* test—may outperform cytology and the HPV16/18 typing test as a triage tool. Furthermore, this study explored the efficacy of combining *PAX1^m/JAM3^m* with HPV16/18 detection and *PAX1^m/JAM3^m* with LBC ≥ ASCUS detection for triage in cervical cancer screening. The results revealed that combining *PAX1^m/JAM3^m* with HPV16/18 detection or LBC ≥ ASCUS detection did not enhance the detection efficacy for CIN3+. This approach may even reduce the specificity for detecting CIN3 + and thus unnecessarily increase the referral rate. According to the subgroup analysis by age, *PAX1^m/JAM3^m* detected was the most effective triage method for women infected with hrHPV. For women under 30 years of age, the AUC for HPV16/18(+) results was greater than that for *PAX1^m/JAM3^m*(+) results (0.737 vs 0.669), but HPV16/18(+) detection had a lower specificity (59.8 vs 96.3). The detection of *PAX1^m/JAM3^m* also outperformed HPV16/18 genotyping test in women over 30 years old. Furthermore, in women aged 30-49 years and those above 50 years, *PAX1^m/JAM3^m*(+) had high sensitivity (65.3% and 79.1%, respectively) and high specificity (90.1% and 86.6%, respectively) for detecting CIN3+. Herzog et al. evaluated the use of a DNA methylation test for the human genes *DPP6*, *RALYL*, and *GSX1* and revealed that DNA methylation was more sensitive than cytology as a triage for detecting invasive cancer and CIN3+ (78% vs 70.7%, respectively).[39] Moreover, the sensitivity of detecting CIN3+was 65% and 83% for women <30 and ≥30 years of age, respectively. The performance of the methylation markers described by Verhoef et al may presumably have been worse in younger women.[40] We observed an age-dependent correlation of *PAX1^m/JAM3^m*(+) results, with sensitivities of 37.5%, 65.3%, and 79.1%, respectively, for detecting CIN3+ among women in the 3 age groups. The lower methylation positivity in CIN3 lesions among younger women, compared to older women, aligns with a shorter duration of associated HPV infection and a likely lower cancer progression risk in these women.[41] More predictive studies on the risk of progression from CIN3 to invasive cancer are necessary. A previous study revealed that a negative FAM19A4/miR12-4 methylation test identified women with CIN2/3 as having the highest chance of clinical regression. The authors suggested that lesions should be addressed only immediately if methylation or HPV16 genotyping results are positive; otherwise, close monitoring should be chosen.[35] Another study demonstrated the high specificity of methylation testing for advanced CIN2/3 lesions in young women, so this test is expected to become a useful tool for guiding clinicians in the management of women with CIN2/3 lesions.[41] This series of studies suggests that in the screening and diagnosis of young women, it is important to focus on the specificity of the tests to identify those who are truly at risk of cancer. While ensuring accurate diagnosis, it is also crucial to consider the preservation of fertility in young women.

Unlike the *MAL* and *miR-124-2* gene methylation triage, which obtained a low positive predictive value (PPV; 19.4% for CIN3+), in the PROHTECT-3 study,[33] the PPV of the *PAX1^m/JAM3^m*(+) in our study was 45.2%, which was greater than the 20.3% in women with HPV16/18(+) and the 13.3% in women with cytology ≥ASCUS. In addition, compared with *PAX1^m/JAM3^m* testing alone, *PAX1^m/JAM3^m* combined with HPV16/18 detection effectively improved the detecting sensitivity of CIN3+ (68.3 vs 88.6, $P < .05$) without reducing the detection efficiency (0.790 vs 0.742, $P > .05$), but this approach also increased the colposcopy referral rate from 16.83% to 45.52%. Therefore, *PAX1^m/JAM3^m*(+) detected has a high accuracy rate and can effectively reduce colposcopy referrals for patients with normal or LSIL pathologic results and prevent panic among women infected with hrHPV. A large international study spanning 5 continents consistently indicated that DNA methylation detection can identify cervical cancer independent of tissue type, HPV genotype, geographic region, and sample type.[42] It has also shown high interlaboratory consistency across several cervical screening sample collection methods.[43] This series of studies provides ample evidence that DNA methylation detection is an effective method for cervical cancer screening.

Two retrospective screening studies further demonstrated that women infected with HPV but exhibit DNA methylation negativity have the same risk of CIN3+ lesions over 14 years in women with concurrent negative cytology results, albeit with a lower risk of developing cervical cancer.[11,44] Moreover, over the following 3 years, women with DNA methylation negativity experienced a significant reduction in the risk of CIN2+.[45] These findings suggest that DNA methylation can be used as a predictive factor for the progression of cervical lesions. Unfortunately, this study did not have corresponding follow-up evidence available and could not confirm the ability of methylation status to predict CIN3+ lesions risk in women.

The shortcoming of this study is that although it enrolled many patients with hrHPV, it was only a single-center study and could not compare the triage efficacy of *PAX1^m/JAM3^m* testing in different regions and populations. Future studies should expand the sample size and include a wider range of populations, and studies in community-based populations could assess the performance of screening methods more

comprehensively. This study also lacked follow-up information for demonstrating the predictive power of the *PAX1*^{m}/*JAM3*^{m} results for the long-term risk of CIN3+, which needs to be confirmed in subsequent studies.

Conclusion

PAX1^{m}/*JAM3*^{m} testing has high specificity for detecting CIN3+ in women infected with hrHPV. As a triage biomarker, its detection efficacy surpasses that of HPV 16/18 genotyping and LBC testing for women with hrHPV infection.

Author contributions

Lei Li conceived the original idea for the study, interpreted the results, carried out the statistical analysis, edited the paper and was the overall guarantor. Linghua Kong and Lei Li obtained ethical approval, contributed to the preparation of the data set, interpreted the results, and contributed to drafts of the paper. Linghua Kong and Xiaoping Xiao performed evaluations of cytology pathology and HPV DNA testing. Xitong Jin, Pei Liu, and Yuligh Liou performed the methylation testing. Jinghe Lang and Linghua Kong contributed to the study design and interpretation of the results and commented on drafts of the paper. Huanwen Wu and Yan You conducted the pathological evaluation and reviewed the original materials. All the authors have approved the final version of the manuscript.

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Conflicts of interest

All the authors declare that they have no conflicts of interest to disclose.

Data availability statement

All data of this study are available upon reasonable request from the corresponding authors.

Clinical trial registration

The registration number is NCT04646954 (*clinicaltrials.gov*, registered on November 26, 2020).

Ethics approval

The Institutional Review Board of Peking Union Medical College Hospital approved this study (No. JS-2380). Informed consent was obtained from the subjects prior to their participation in the study.

Patient consent

Consent for publication has been obtained from all patients.

Supplementary material

Supplementary material is available at *The Oncologist* online.

References

1. Bouvard V, Wentzensen N, Mackie A, et al. The IARC perspective on cervical cancer screening. *N Engl J Med*. 2021;385:1908-1918. https://doi.org/10.1056/NEJMsr2030640
2. Das M. WHO launches strategy to accelerate elimination of cervical cancer. *Lancet Oncol*. 2021;22:20-21. https://doi.org/10.1016/S1470-2045(20)30729-4
3. Ronco G, Dillner J, Elfström KM, et al; International HPV screening working group. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet*. 2014;383:524-532. https://doi.org/10.1016/S0140-6736(13)62218-7
4. Castle PE, Schiffman M, Wheeler CM, Solomon D. Evidence for frequent regression of cervical intraepithelial neoplasia-grade 2. *Obstet Gynecol*. 2009;113:18-25. https://doi.org/10.1097/aog.0b013e31818f5008
5. Wentzensen N, Schiffman M, Palmer T, Arbyn M. Triage of HPV positive women in cervical cancer screening. *J Clin Virol*. 2016;76:S49-S55. https://doi.org/10.1016/j.jcv.2015.11.015
6. Bergeron C, Giorgi-Rossi P, Cas F, et al. Informed cytology for triaging HPV-positive women: substudy nested in the NTCC randomized controlled trial. *J Natl Cancer Inst*. 2015;107:dju423. https://doi.org/10.1093/jnci/dju423
7. Bonde J, Floore A, Ejegod D, et al. Methylation markers FAM19A4 and miR124-2 as triage strategy for primary human papillomavirus screen positive women: a large European multicenter study. *Int J Cancer*. 2021;148:396-405. https://doi.org/10.1002/ijc.33320
8. Li D, Zhang L, Fu J, et al. Discovery and validation of tissue-specific DNA methylation as noninvasive diagnostic markers for colorectal cancer. *Clin Epigenetics*. 2022;14:102. https://doi.org/10.1186/s13148-022-01312-9
9. Clarke MA, Wentzensen N, Mirabello L, et al. Human papillomavirus DNA methylation as a potential biomarker for cervical cancer. *Cancer Epidemiol Biomarkers Prev*. 2012;21:2125-2137. https://doi.org/10.1158/1055-9965.EPI-12-0905
10. De Strooper LMA, van Zummeren M, Steenbergen RDM, et al. CADM1, MAL and miR124-2 methylation analysis in cervical scrapes to detect cervical and endometrial cancer. *J Clin Pathol*. 2014;67:1067-1071. https://doi.org/10.1136/jclinpath-2014-202616
11. De Strooper LMA, Berkhof J, Steenbergen RDM, et al. Cervical cancer risk in HPV-positive women after a negative FAM19A4/mir124-2 methylation test: a post hoc analysis in the POBASCAM trial with 14 year follow-up. *Int J Cancer*. 2018;143:1541-1548. https://doi.org/10.1002/ijc.31539
12. Steenbergen RDM, Snijders PJF, Heideman DAM, Meijer CJLM. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. *Nat Rev Cancer*. 2014;14:395-405. https://doi.org/10.1038/nrc3728
13. von Knebel Doeberitz M, Prigge E-S. Role of DNA methylation in HPV associated lesions. *Papillomavirus Res*. 2019;7:180-183. https://doi.org/10.1016/j.pvr.2019.03.005

14. Lorincz AT. Virtues and weaknesses of DNA methylation as a test for cervical cancer prevention. *Acta Cytol*. 2016;60:501-512. https://doi.org/10.1159/000450595
15. Adcock R, Nedjai B, Lorincz AT, et al; New Mexico HPV Pap Registry Steering Committee. DNA methylation testing with S5 for triage of high-risk HPV positive women. *Int J Cancer*. 2022;151:993-1004. https://doi.org/10.1002/ijc.34050
16. Salta S, Lobo J, Magalhães B, Henrique R, Jerónimo C. DNA methylation as a triage marker for colposcopy referral in HPV-based cervical cancer screening: a systematic review and meta-analysis. *Clin Epigenetics*. 2023;15:125. https://doi.org/10.1186/s13148-023-01537-2
17. Kaliff M, Lillsunde Larsson G, Helenius G, Karlsson MG, Bergengren L. Full genotyping and FAM19A4/miR124-2 methylation analysis in high-risk human papillomavirus-positive samples from women over 30 years participating in cervical cancer screening in Örebro, Sweden. *PLoS One*. 2022;17:e0274825. https://doi.org/10.1371/journal.pone.0274825
18. Kan YY, Liou YL, Wang HJ, et al. PAX1 methylation as a potential biomarker for cervical cancer screening. *Int J Gynecol Cancer*. 2014;24:928-934. https://doi.org/10.1097/IGC.0000000000000155
19. Liou YL, Zhang TL, Yan T, et al. Combined clinical and genetic testing algorithm for cervical cancer diagnosis. *Clin Epigenetics*. 2016;8:66. https://doi.org/10.1186/s13148-016-0232-3
20. Kong L, Wang L, Wang Z, et al. DNA methylation for cervical cancer screening: a training set in China. *Clin Epigenetics*. 2020;12:91. https://doi.org/10.1186/s13148-020-00885-7
21. Kong L, Wang L, Wang Z, et al. Cytological DNA methylation for cervical cancer screening: a validation set. *Front Oncol*. 2023;13:1181982. https://doi.org/10.3389/fonc.2023.1181982
22. Rao A, Young S, Erlich H, et al. Development and characterization of the cobas human papillomavirus test. *J Clin Microbiol*. 2013;51:1478-1484. https://doi.org/10.1128/JCM.03386-12
23. Nayar R, Wilbur DC. The pap test and Bethesda 2014. *Cancer Cytopathol*. 2015;123:271-281. https://doi.org/10.1002/cncy.21521
24. Fontham ETH, Wolf AMD, Church TR, et al. Cervical cancer screening for individuals at average risk: 2020 guideline update from the American Cancer Society. *CA Cancer J Clin*. 2020;70:321-346. https://doi.org/10.3322/caac.21628
25. Perkins RB, Wentzensen N, Guido RS, Schiffman M. Cervical cancer screening: a review. *JAMA*. 2023;330:547-558. https://doi.org/10.1001/jama.2023.13174
26. Li N, Hu Y, Zhang X, et al. DNA methylation markers as triage test for the early identification of cervical lesions in a Chinese population. *Int J Cancer*. 2021;148:1768-1777. https://doi.org/10.1002/ijc.33430
27. McBride E, Tatar O, Rosberger Z, et al. Emotional response to testing positive for human papillomavirus at cervical cancer screening: a mixed method systematic review with meta-analysis. *Health Psychol Rev*. 2021;15:395-429. https://doi.org/10.1080/17437199.2020.1762106
28. Uner FO, Korukcu O. A prevalence and psychometric study on fear of cancer in women with abnormal cervical cytology undergoing colposcopy. *Psychooncology*. 2020;29:1850-1855. https://doi.org/10.1002/pon.5504
29. Zhang R, Shen C, Zhao L, et al. Dysregulation of host cellular genes targeted by human papillomavirus (HPV) integration contributes to HPV-related cervical carcinogenesis. *Int J Cancer*. 2016;138:1163-1174. https://doi.org/10.1002/ijc.29872
30. Da Silva MLR, De Albuquerque B, Allyrio T, et al. The role of HPV-induced epigenetic changes in cervical carcinogenesis (Review). *Biomed Rep*. 2021;15:60. https://doi.org/10.3892/br.2021.1436
31. Soto D, Song C, McLaughlin-Drubin ME. Epigenetic alterations in human papillomavirus-associated cancers. *Viruses*. 2017;9:248. https://doi.org/10.3390/v9090248
32. Zamani S, Sohrabi A, Hosseini SM, Rahnamaye-Farzami M, Akbari A. Deregulation of miR-21 and miR-29a in cervical cancer related to HPV infection. *Microrna*. 2019;8:110-115. https://doi.org/10.2174/2211536607666181017124349
33. Verhoef VM, Bosgraaf RP, van Kemenade FJ, et al. Triage by methylation-marker testing versus cytology in women who test HPV-positive on self-collected cervicovaginal specimens (PROHTECT-3): a randomised controlled non-inferiority trial. *Lancet Oncol*. 2014;15:315-322. https://doi.org/10.1016/S1470-2045(14)70019-1. S1470-2045(14)70019-1 [pii]
34. Dick S, Vink FJ, Heideman DAM, et al. Risk-stratification of HPV-positive women with low-grade cytology by FAM19A4/miR124-2 methylation and HPV genotyping. *Br J Cancer*. 2022;126:259-264. https://doi.org/10.1038/s41416-021-01614-4
35. Kremer WW, Dick S, Heideman DAM, et al. Clinical regression of high-grade cervical intraepithelial neoplasia is associated with absence of FAM19A4/miR124-2 DNA methylation (CONCERVE Study). *J Clin Oncol*. 2022;40:3037-3046. https://doi.org/10.1200/JCO.21.02433
36. Boers A, Wang R, van Leeuwen RW, et al. Discovery of new methylation markers to improve screening for cervical intraepithelial neoplasia grade 2/3. *Clin Epigenetics*. 2016;8:29. https://doi.org/10.1186/s13148-016-0196-3
37. Louvanto K, Aro K, Nedjai B, et al. Methylation in predicting progression of untreated high-grade cervical intraepithelial neoplasia. *Clin Infect Dis*. 2020;70:2582-2590. https://doi.org/10.1093/cid/ciz677
38. Reuter C, Preece M, Banwait R, et al. Consistency of the S5 DNA methylation classifier in formalin-fixed biopsies versus corresponding exfoliated cells for the detection of pre-cancerous cervical lesions. *Cancer Med*. 2021;10:2668-2679. https://doi.org/10.1002/cam4.3849
39. Herzog C, Sundström K, Jones A, et al. DNA methylation-based detection and prediction of cervical intraepithelial neoplasia grade 3 and invasive cervical cancer with the WID™-qCIN test. *Clin Epigenetics*. 2022;14:150. https://doi.org/10.1186/s13148-022-01353-0
40. Beiersdorf J, Scheungraber C, Wunsch K, et al. Combined assessment of 3q26 amplification and promoter methylation in patients with high grade cervical lesions show age specific differences. *Genes Chromosomes Cancer*. 2020;59:168-177. https://doi.org/10.1002/gcc.22818
41. Vink FJ, Meijer C, Hesselink AT, et al. FAM19A4/miR124-2 methylation testing and human papillomavirus (HPV) 16/18 genotyping in HPV-positive women under the age of 30 years. *Clin Infect Dis*. 2023;76:e827-e834. https://doi.org/10.1093/cid/ciac433
42. Vink FJ, Meijer CJLM, Clifford GM, et al. FAM19A4/miR124-2 methylation in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Int J Cancer*. 2020;147:1215-1221. https://doi.org/10.1002/ijc.32614
43. Floore A, Hesselink A, Oštrbenk A, et al. Intra- and inter-laboratory agreement of the FAM19A4/mir124-2 methylation test: results from an international study. *J Clin Lab Anal*. 2019;33:e22854. https://doi.org/10.1002/jcla.22854
44. Dick S, Kremer WW, De Strooper LMA, et al. Long-term CIN3+ risk of HPV positive women after triage with FAM19A4/miR124-2 methylation analysis. *Gynecol Oncol*. 2019;154:368-373. https://doi.org/10.1016/j.ygyno.2019.06.002
45. Zhang L, Zhao X, Hu S, et al. Triage performance and predictive value of the human gene methylation panel among women positive on self-collected HPV test: results from a prospective cohort study. *Int J Cancer*. 2022;151:878-887. https://doi.org/10.1002/ijc.34041