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Triage accuracy of a six-gene methylation detection for high-risk HPV-positive Chinese women

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Abstract

This study aimed to evaluate the triage accuracy of a six-gene methylation detection test in high-risk human papillomavirus-positive women in China. A total of 369 cervical exfoliated cell samples were collected from high-risk human papillomavirus-positive women undergoing cervical cancer screening at Nanjing Drum Tower Hospital from October 2018 to August 2019. The methylation status of six gene was assessed using real-time methylation-specific polymerase chain reaction. The triage accuracy for cervical intraepithelial neoplasia grade 2 and 3 was compared with that of a commercial methylation kit, cervical cytology, and human papillomavirus types 16/18 tests using receiver operating characteristic curves. Additionally, the effectiveness of the six-gene test for triaging cervical intraepithelial neoplasia grade 2+ and 3+ was validated in 215 samples collected between January and November 2020 and compared with p16/Ki-67 dual-staining cytology. The results demonstrated a significant increase in the positive methylation rate with the severity of cervical intraepithelial neoplasia (P<0.001). This six-gene methylation pattern holds promise for improving triage accuracy in cervical cancer screening for high-risk human papillomavirus-positive women *(Afr J Reprod Health 2025; 29 [7]: 137-147).*

Keywords: Six-gene methylation detection; high-risk HPV; cervical cancer; triage strategy

Résumé

Cette étude visait à évaluer la précision du triage d'un test de détection de la méthylation de six gènes chez des femmes chinoises à haut risque positives au papillomavirus humain. Au total, 369 échantillons de cellules exfoliées cervicales ont été prélevés chez des femmes à haut risque positives au papillomavirus humain, soumises à un dépistage du cancer du col de l'utérus à l'hôpital Nanjing Drum Tower, d'octobre 2018 à août 2019. Le statut de méthylation de six gènes a été évalué par PCR en temps réel spécifique de la méthylation. La précision du triage pour les néoplasies intraépithéliales cervicales de grade 2 et 3 a été comparée à celle d'un kit de méthylation commercial, de la cytologie cervicale et des tests de détection des papillomavirus humains de types 16 et 18 à l'aide des courbes caractéristiques d'efficacité du récepteur. De plus, l'efficacité du test à six gènes pour le triage des néoplasies intraépithéliales cervicales de grade 2+ et 3+ a été validée sur 215 échantillons prélevés entre janvier et novembre 2020 et comparée à la cytologie à double coloration p16/Ki-67. Les résultats ont démontré une augmentation significative du taux de méthylation positive avec la sévérité de la néoplasie intraépithéliale cervicale (p < 0,001). Ce profil de méthylation à six gènes est prometteur pour améliorer la précision du triage dans le dépistage du cancer du col de l'utérus chez les femmes à haut risque positif au papillomavirus humain *(Afr J Reprod Health 2025; 29 [7]: 137-147).*

Mots-clés: Détection de la méthylation à six genes; HPV à haut risqué; cancer du col de l'utérus; stratégie de triage

Introduction

Cervical cancer (CC) shows increasing trends in prevalence and mortality in China, and its onset age is becoming younger[1-3]. It severely endangers women's health. In 2020, statistics reported 110,000 newly onset and 60,000 deaths of CC in China, accounting for 18.2% and 17.3% of global estimates, respectively[4,5].

Persistent high-risk human papillomavirus (hrHPV) infection is a prerequisite for the development of cervical cancer and precancerous

lesions[6,7]. The WHO Guideline for Screening and Treatment of Cervical Pre-Cancer Lesions for Cervical Cancer Prevention recommend the HPV DNA testing as the preferred method for primary CC screening[8]. It provides high sensitivity and negative predictive value in screening CC, but is limited due to the low specificity. Therefore, triage strategies with high sensitivity and specificity for HPV-positive women are urgently needed to reduce unnecessary colposcopy referrals and invasive procedures[9-11]. At present, cervical cytology and oncogenic HPV types 16/18 test are the mainstream methods for triaging the hrHPV population. Cervical cytology, although subjective, is highly dependent on the clinical experiences of pathologists[12-14].

Despite the high sensitivity in identifying high-grade cervical intraepithelial neoplasia (CIN) and CC, the p16/Ki-67 dual-staining cytology has a primary drawback of generating a high number of false positives[15-21].

Latest evidence shows increased methylation levels of the DLX1, ITGA4, RXFP3, SOX17 and ZNF671 genes with severity of cervical lesions, suggesting that the methylation levels in these genes are potential biomarkers for triaging hrHPV-positive women[22,23]. Hypermethylation status of genes associated with cervical cancer is associated with race and living environment[24-26]. In the present study, we designed a six-gene methylation detection and validated its performance in identifying high-grade CIN in Chinese women by comparing with existing triage strategies.

Methods

Subjects

Approved by the ethics committee (Approval No. 2020-333-02), written informed consent was provided by all subjects. From October 2018 to August 2019, 369 cervical exfoliated cell samples were collected from hrHPV-positive women undergoing cervical cancer (CC) screening in the Health Management Center and Gynecological Outpatient Department of the Nanjing Drum Tower Hospital. Dataset validation consisted of 215 cervical exfoliated cell samples of the same population obtained in the institution from January 2020 to November 2020. The subjects were Chinese female aged 21 to 65 years with intact uterine cervix and documented sexual activity history.

Classification of cervical intraepithelial neoplasia (CIN)

CIN was classified into grade 1-3 based on the severity of abnormal cell growth in the cervix. Briefly, the condition of abnormal cells affecting up to one-third, two-thirds and the entire thickness of the cervical lining was considered as CIN1, 2 and 3, respectively [DOI: 10.3310/hta22540].

Real-time methylation-specific PCR (MSP)

Cervical exfoliated cells samples were first treated with a bisulfite reagent and amplified. A PCR mixture involving bisulfite-converted DNA, SYBR Green PCR Master Mix and primers was prepared to the real-time PCR.

Quantitative fluorescence PCR (QF-PCR)

A commercial methylation detection kit (Shanghai GeneoDx Biotech, Co., Ltd) was used to detect DNA methylation statue of the human ASTN1, DLX1, ITGA4, RXFP3, SOX17 and ZNF671 genes according to the manufacture's recommendation. Cervical exfoliated cells collected from a scrape sample were immediately preserved in cell preservation solution and subjected to QF-PCR on an ABI 7500 real-time fluorescence PCR system.

Cervical cytology

A speculum was used to access the cervix, and cervical cells were collected by gently scraping the ectocervix and placed on a slide (Pap smear). It was then sent to the Laboratory Department of Nanjing Drum Tower Hospital for microscopic examination. A positive finding of greater than or equal to atypical squamous cells of undetermined significance (ASCUS), a referral to colposcopy was necessary.

HPV 16/18 testing

Cervical tissues ahead, astern, and to the left and right of the squamocolumnar junction (SCJ) were collected and sent to the Laboratory Department of

Nanjing Drum Tower Hospital for testing HPV 16/18.

P16/Ki-67 dual-staining

Cervical tissue was processed for a tissue slide. It was incubated with a cocktail of primary antibodies against both p16 and Ki-67 using the Roche CINtec® PLUS Cytology Kit. After washing, the slide was incubated with DAB for p16 and red chromogen for Ki-67. Following counterstaining with hematoxylin, dual-staining of p16/Ki-67 was captured under a fluorescent microscope.

Statistical analysis

SPSS 22.0 and Medcalc 16.8.4 were used for the statistical analysis. Logistic regression was performed to determine the methylation patterns in the six genes using the equation: logit(P) = ln[P / (1 - P)]. Odds ratio (OR) and corresponding 95% cervical interval (CI) were calculated. The receiver operating characteristic (ROC) curves were plotted to identify the diagnostic potential, with the calculations of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the curve (AUC). A significant difference was determined by $p<0.05$.

Ethical considerations

This study was approved by the Institutional Review Board of Nanjing University Affiliated Drum Tower Hospital (Approval No.:2018-011-02; Date:July 18, 2018). Written informed consent was obtained from all participants prior to any study procedures.

Results

A linear correlation between the six-gene positive methylation rates and the severity of CIN

The mean age was 42.2±10.1, with a range of 23 to 65 years. Based on the pathological diagnosis, there were 4 cases of squamous cell carcinoma of the cervix (SCC), 37 cases of CIN3, 32 cases of CIN2, 63 cases of CIN1, and 233 healthy cases. As shown in Figure 1, the positive methylation rate in the six genes significantly increased with the severity of CIN ($\chi2$=107.60, 60.40, 128.84, 90.68, 111.85 and 188.60, respectively; all $P<0.001$) (Figure 1). Among them, methylation levels of the Distal-less Homeobox 1 (DLX1), Integrin Subunit Alpha 4 (ITGA4) and zinc finger protein 671 (ZNF671) genes achieved 100% in SCC. In comparison to other genes, the methylation levels of the ZNF671 gene ranged the highest in CIN2 and CIN3, but remained in low in CIN1 and healthy cases. In contrast, the methylation pattern of the RXFP3 showed the lowest levels in CIN2 and CIN3. Overall, methylation patterns of the six genes possessed subtle variations in CIN and CC, and all of them had significantly increased levels with the severity of cervical lesions.

Correlation of methylation levels in the six genes with CIN and CC

Among 369 hrHPV-positive women, pathology determined CIN2+ (including CIN2, CIN3 and SCC) in 73 cases, and CIN3+ (including CIN3 and SCC) in 41 cases. As shown in Table 1, methylation in the ZNF671 gene was positive in 53 (72.60%) CIN2+ lesions (OR 46.38, 95% CI 22.57, 95.27), and 36 (87.81%) CIN3+ lesions (OR 64.36, 95% CI 23.63, 175.35), ranking the top of the six genes in distinguishing CIN and CC (Table 1). Positivity for the RXFP3 gene methylation was the lowest among the six genes in both CIN2+ (34.25%, OR 25.17, 95% CI 9.82, 64.57) and CIN3+ lesions (43.90%, OR 18.96, 95% CI 8.27, 43.47).

A six-gene methylation pattern

With CIN2+ lesions as the endpoint, the six genes (X_A, *ASTN1*; X_D, *DLX1*; X_I, *ITGA4*; X_R, *RXFP3*; X_S, *SOX17*; X_Z, *ZXNF671*) were incorporated into the Logistic equation., We obtained a six-gene methylation pattern as follows: $\text{logit}(P) = -2.80^{**}+1.25^{*}X_A-1.50^{*}X_D+0.70X_I+1.83^{*}X_R+0.96X_S+3.28^{**}X_Z$. As shown in Table 2, using the six-gene methylation pattern, ROC curves reported an AUC of 0.884 (95% CI 0.830, 0.937) in distinguishing CIN2+ lesions, with a perfect sensitivity of 100%, but an extremely low specificity of 5.4%. The colposcopy referral rate was 95.7% (Table 2).

Table 1: Positivity for the six-gene methylation levels in pathologically confirmed CIN and CC

Genes	CIN2+ (n=73) Methylation positivity (n, %)	OR (95% CI)	CIN3+ (n=41) Methylation positivity (n, %)	OR (95% CI)
ASTN1	41 (56.16%)	17.68 (9.25-33.80)	26 (63.41%)	14.51 (7.02-29.98)
DLX1	32 (43.84%)	6.44 (3.57-11.62)	23 (56.10%)	8.94 (4.45-17.98)
ITGA4	27 (36.99%)	42.85 (14.33-128.01)	20 (48.78%)	27.45 (11.64-64.74)
RXFP3	25 (34.25%)	25.17 (9.82-64.57)	18 (43.90%)	18.96 (8.27-43.47)
SOX17	33 (45.21%)	26.31 (11.73-59.01)	22 (53.66%)	17.83 (8.32-38.22)
ZNF671	53 (72.60%)	46.38 (22.57-95.27)	36 (87.81%)	64.36 (23.63-175.35)

CIN, cervical intraepithelial neoplasia; CC, cervical cancer; OR, odds ratio; CI, confidence interval.

Table 2: The diagnostic performance of the six-gene methylation pattern

Diagnostic performance	Values
Sensitivity (%)	100
Specificity (%)	5.4
PPV (%)	20.7
AUC (95% CI)	0.884 (0.830, 0.937)
Colposcopy referral rate (%)	95.7

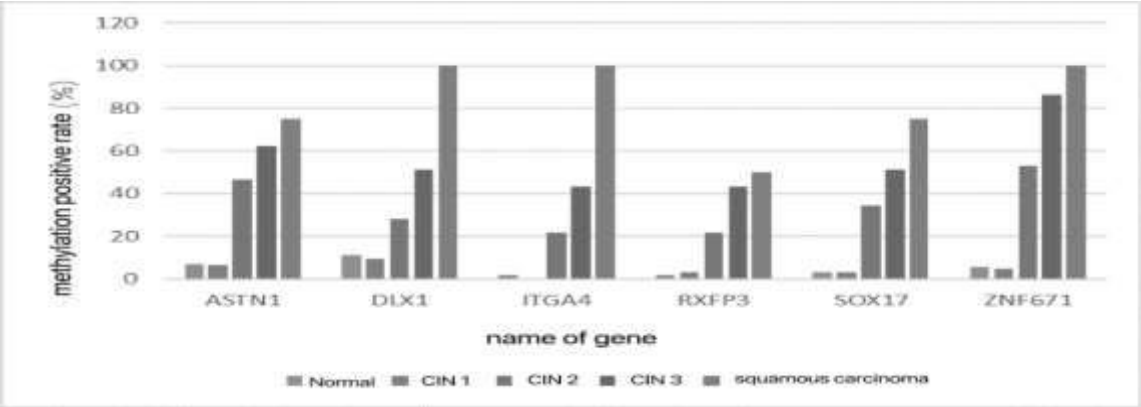

Figure 1: Distribution of methylation patterns of the ASTN1, DLX1, ITGA4, RXFP3, SOX17 and ZNF671 genes in healthy cases, CIN1, CIN2, CIN3 and SCC.

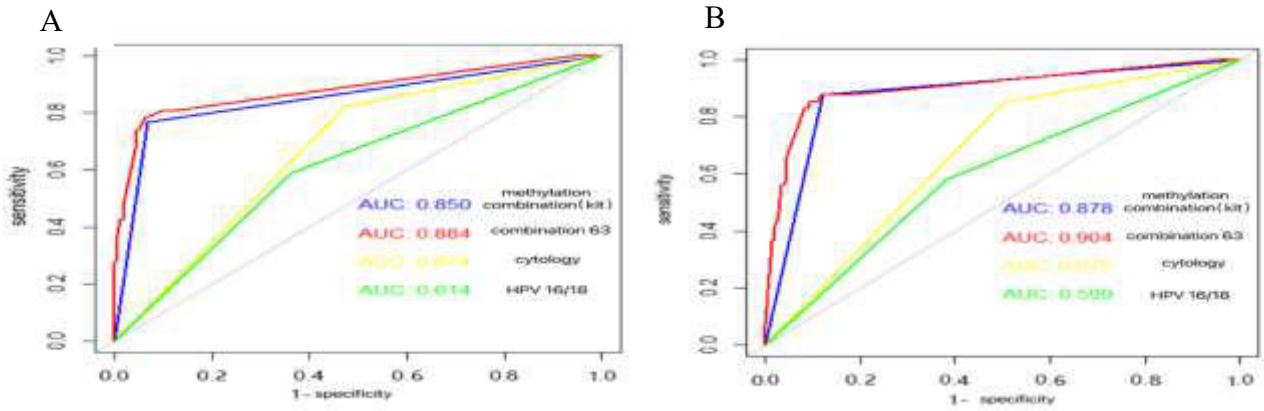

Figure 2: ROC curves of the six-gene methylation detection, commercial methylation detection kit, cervical cytology and HPV 16/18 testing in distinguishing CIN2+ (A) and CIN3+ lesions (B).

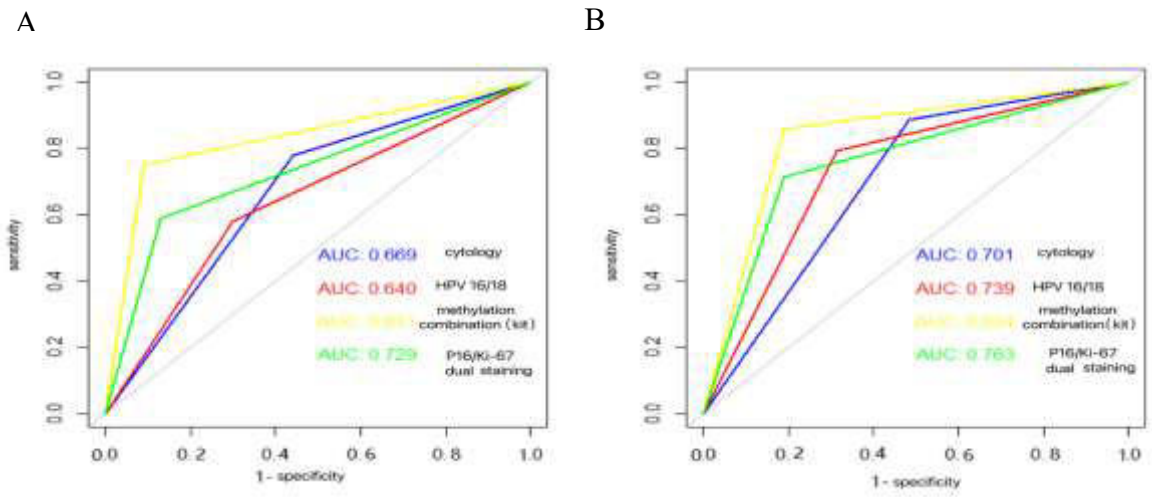

Figure 3: ROC curves of the commercial methylation detection kit, p16/Ki-67 dual-staining, cervical cytology and HPV 16/18 testing in distinguishing CIN2+ (A) and CIN3+ lesions (B).

The potential of the six-gene methylation detection as a triage strategy for hrHPV-positive women

To test the potential of the six-gene methylation detection as a triage strategy for hrHPV-positive women, we compared its performance with existing tools of the commercial methylation detection kit, cervical cytology and HPV 16/18 testing. With CIN2+ lesions as the endpoint, the six-gene methylation detection owned the highest sensitivity (100%) in triaging CIN2+, but the lowest specificity (5.4%). The largest AUC was detected in the six-gene methylation detection (0.884), followed by the commercial methylation detection kit (0.850), cervical cytology (0.674) and HPV 16/18 testing (0.614). However, the six-gene methylation detection exerted a poor triage effect on hrHPV-positive women, with the colposcopy referral rate of 95.7%, and 4.8 colposcopy referrals required to detect one CIN2+ case (Table 3, Figure 2A). Only 1.4 colposcopy referrals were essential to detect one CIN2+ lesion using the commercial methylation detection kit.

Similar results were obtained when CIN3+ lesions were taken as the endpoint. The six-gene methylation detection was able to identify CIN3+ lesions with a 100% of sensitivity, although its septicity was as low as 4.9%. It possessed the largest AUC (0.895) than the commercial methylation detection kit (0.878), cervical cytology (0.675) and HPV 16/18 testing (0.599). The six-gene methylation detection, commercial methylation detection kit, cervical cytology and HPV 16/18 testing required 8.6, 2.1, 5.7 and 6.9 colposcopy referrals to detect one CIN3+ lesion (Table 4, Figure 2B).

Detection of the methylation levels in the six genes contributes to triage hrHPV-positive women

Although the six-gene methylation detection was unable to reduce the colposcopy referral rate compared with the existing triage strategies, measurements of methylation levels in the six genes using the commercial kit performed well in triaging hrHPV-positive women. It was capable of reducing the number of unnecessary colposcopies and treatments for low-risk individuals. 215 cervical exfoliated cell samples were collected from hrHPV-positive women screened for CC for validating the triage effect of methylation detection of the six genes. The mean age of the women was 41.9±10.7 (21-65) years. Pathologically, there were three cases of SCC, one case of adenocarcinoma in situ (AIS) of the cervix, 31 cases of CIN3, 33 cases of CIN2, 27 cases of CIN1 and 120 healthy cases. The absence of triage strategies resulted in a universal colposcopy referral rate (100%).

Table 3: Clinical performance of the six-gene methylation detection in triaging hrHPV-positive women with the endpoint of CIN2+

	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**AUC (95% CI)**	**Colposcopy referral rate (%)**	**Colposcopy referrals to detect one CIN2+ case**
Six-gene methylation detection	100.0	5.4	20.7	100.0	0.884 (0.830, 0.937)	95.7	4.8
Commercial methylation detection kit	76.7	93.2	73.7	94.2	0.850 (0.790, 0.909）	20.6	1.4
Cervical cytology (≥ASCUS)	82.2	52.7	30.0	92.3	0.674 (0.610, 0.739)	54.2	3.3
HPV 16/18 testing	58.9	63.9	26.6	87.5	0.614 (0.532, 0.696）	40.3	3.8

hrHPV, high-risk human papillomavirus; CIN, cervical intraepithelial neoplasia; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve; CI, confidence interval; ASCUS, atypical squamous cells of undetermined significance.

Table 4: Clinical performance of the six-gene methylation detection in triaging hrHPV-positive women with the endpoint of CIN3+

	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**AUC (95% CI)**	**Colposcopy referral rate (%)**	**Colposcopy referrals to detect one CIN3+ case**
Six-gene methylation detection	100.0	4.9	11.6	100.0	0.895 (0.845, 0.902)	95.7	8.6
Commercial methylation detection kit	87.8	87.8	47.4	98.3	0.878 (0.817, 0.939)	20.6	2.1
Cervical cytology (≥ASCUS)	85.4	49.7	17.5	96.5	0.675 (0.598, 0.753)	54.2	5.7
HPV 16/18 testing	58.1	61.7	14.5	92.9	0.599 (0.493, 0.705)	40.3	6.9

hrHPV, high-risk human papillomavirus; CIN, cervical intraepithelial neoplasia; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve; CI, confidence interval; ASCUS, atypical squamous cells of undetermined significance.

Table 5: Triage effect of the methylation detection in the six genes on CIN2+ from hrHPV-positive women

	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**AUC (95% CI)**	**Colposcopy referral rate (%)**	**Colposcopy referrals to detect one CIN2+ case**
Commercial methylation detection kit	75.0	91.2	79.7	88.7	0.831 (0.764, 0.897)	29.8	1.3
P16/Ki-67 dual-staining cytology	58.8	87.1	67.8	82.1	0.729 (0.651, 0.808)	27.4	1.5
Cervical cytology (≥ASCUS)	77.9	55.8	44.9	84.5	0.669 (0.593, 0.744)	54.9	2.2
HPV 16/18 testing	58.0	70.0	42.7	81.3	0.640 (0.548, 0.732)	37.8	2.4

CIN, cervical intraepithelial neoplasia; hrHPV, high-risk human papillomavirus; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve; CI, confidence interval; ASCUS, atypical squamous cells of undetermined significance.

Table 6: Triage effect of the methylation detection in the six genes on CIN3+ from hrHPV-positive women

	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**AUC (95% CI)**	**Colposcopy referral rate (%)**	**Colposcopy referrals to detect one CIN3+ case**
Commercial methylation detection kit	85.7	81.1	46.9	96.7	0.834 (0.759, 0.909)	29.8	2.1
P16/Ki-67 dual-staining cytology	71.4	81.1	42.4	93.6	0.763 (0.669, 0.856)	27.4	2.4
Cervical cytology (≥ASCUS)	88.6	51.7	26.3	95.9	0.701 (0.617, 0.785)	54.9	3.8
HPV 16/18 testing	79.2	68.6	27.9	95.5	0.739 (0.635, 0.843)	37.8	3.6

CIN, cervical intraepithelial neoplasia; hrHPV, high-risk human papillomavirus; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve; CI, confidence interval; ASCUS, atypical squamous cells of undetermined significane

Comparative analysis revealed that methylation profiling of six target genes using the standardized commercial assay demonstrated superior performance, yielding a significantly reduced colposcopy referral rate (29.8%) compared to conventional cervical cytology (54.9%; p<0.001) and HPV16/18 genotyping (37.8%; p=0.032). This performance was comparable to the established p16/Ki-67 dual-staining cytology (27.4%; p=0.412) (Table 5). Notably, the methylation-based approach exhibited the highest diagnostic accuracy among evaluated strategies, as evidenced by its leading specificity, positive predictive value (PPV), negative predictive value (NPV) and area under the curve (AUC). Furthermore, this method demonstrated exceptional clinical efficiency, requiring only 1.3 colposcopy referrals per confirmed CIN2+ case (Table 5, Figure 3A).
Similarly, the triage effect of detecting methylation levels of the six genes was excellent in identifying CIN3+ from hrHPV-positive women, showing the highest sensitivity, PPV, NPV, and AUC among the four strategies. It presented the same specificity and comparable colposcopy referral rate with those of the p16/Ki-67 dual-staining (Table 6, Figure 3B). Clinical detection of the methylation levels in the six genes significantly reduced the number of colposcopy referrals to detect a single CIN3+ lesion, indicating that it was an effective triage effect to avoid unnecessary colposcopies and biopsies.

Discussion

Cervical cancer is the fourth most common gynecological malignancy and the second leading cause of cancer-associated female mortality globally. It is closely linked to HPV infection, particularly HPV16 and HPV18. HrHPV testing is currently a mainstream method for screening CC, although its high sensitivity can lead to a high rate of false positives, unnecessary colposcopies and overtreatment[27,28]. An effective triage strategy for identifying the high-risk populations of CC and precancerous lesions is particularly important in undeveloped areas with poor accesses to medical resources.DNA methylation is a key factor in the progression of CIN to cervical cancer. At present, more than 100 methylation markers have been detected in cervical tissue, and 10 of them significantly increase in high-grade cervical lesions and cervical cancer[29]. Previous data show a promising DNA methylation signature involving the *ASTN1*, *DLX1*, *ITGA4*, *RXFP3*, *SOX17* and *ZNF671* genes in triaging hrHPV-positive women. Its performance in detecting CC and precancerous stages has been widely validated in women from Europe and the North India[22,23]. In addition to the triage effect, methylation markers can be used to predict the progression of CC and its recurrence[30].

So far, the role of the six-gene methylation in triaging Chinese women with hrHPV-positive infection remains largely unclear. In our study, the positive methylation rate in the six genes significantly increased with the severity of CIN in 369 cervical exfoliated cell samples collected from hrHPV-positive women in China. A six-gene methylation pattern was found to distinguish CIN2+ lesions with a perfect sensitivity of 100%. However, the specificity was only 5.4%. As a triage strategy, the performance of the self-designed six-gene methylation detection was inferior to the existing methods in identifying CIN2+ and CIN3+ lesions. Overall, the six-gene methylation detection was able to sensitively identify precancerous lesions and CC, but limited by the increased false positives, patient anxiety, healthcare resource utilization, and potential for missed CC due to the low specificity[31]. Compared with the cervical cytology and HPV 16/18 testing, the commercial methylation detection kit of detecting the DNA methylation (Shanghai GeneoDx Biotech, Co., Ltd) was the most superior tool to distinguish CIN2+ and CIN3+ lesions, with the highest specificity, PPV, NPV and AUC. Cytology is a conventional triage method after an HPV test, offering a detailed assessment of abnormal cervical cells in HPV-positive individuals and a better identification of risky women requiring further colposcopies[32]. It is a cost-effective examination with a high specificity, but the interpretation of cytological results was highly dependent on the clinical experiences[33]. Spence *et al*. reported that 20%-55% of women who finally develop CC have false-negative cytological findings within the past 6 years before a clear diagnosis is made[34]. An initial HPV screening is a

reliable triage test that identifies women at high risk of precancerous lesions and CC. The high sensitivity (95%) of an HPV testing makes it ideal for primary CC screening[35]. However, an HPV test is designed to detect potential HPV infection, which is closely but not identical to CC.

Drawbacks of HPV testing also include the low PPV, expensive laboratory infrastructure and long detection time[36]. Thomsen et al. demonstrated that HPV-based screening offers an increased identification of CIN3+ by 90% but 3-fold increase in the colposcopy referrals in comparison to cervical cytology[37]. Our data consistently revealed the inferior performances of cervical cytology and HPV 16/18 testing in triaging CIN2+ and CIN3+ than the methylation detection in the six genes.

Although the self-designed six-gene methylation detection did not perform ideally in triaging precancerous lesions and CC, the commercial methylation detection kit exerted an excellent triage effect. We later examined its efficacy on triaging 215 cervical exfoliated cell samples from highrHPV-positive women. As expected, it presented the same specificity and comparable colposcopy referral rate with those of the p16/Ki-67 dual-staining. Through a simultaneous identification of two biomarkers associated with cervical cancer, p16/Ki-67 dual-staining allows for more accurate identification of women who require further colposcopies[38]. Supported by our data, only 1.5 and 2.4 colposcopy referrals were essential to detect a single CIN2+ and CIN3+ case, respectively. A low colposcopy referral rate potentially reduces unnecessary biopsies, alleviates negative emotions of affected individuals and prevents the waste of medical resources[39]. The methylation detection of the six genes even outperformed the p16/Ki-67 dual-staining, showing a smaller number of colposcopy referral to detect either a CIN2+ or CIN3+ case.

Overall, methylation statue of the six genes is closely linked with CIN and cervical cancer. Although the self-designed six-gene methylation detection performed less efficacy on triaging precancerous lesions and CC from hrHPV-positive women in China, the existing commercial kit did perform an excellent triage effect. Our results demonstrate that methylation level of the six genes (ASTN1, DLX1, ITGA4, RXFP3, SOX17, and ZNF671) was closely linked with CIN and CC in Chinese women. Although the self-designed six-gene methylation detection performed less efficaciously in triaging precancerous lesions and CC from hrHPV-positive Chinese women, the commercial six-gene methylation detection kit did perform an excellent triage effect in the same population. Nevertheless, this six-gene methylation pattern demonstrates potential for enhancing triage accuracy in cervical cancer screening for hrHPV-positive Chinese women

Study strengths and limitations

This study has several notable strengths. First, the development of a six-gene methylation panel represents a molecular advancement, providing improved specificity for CIN2+ detection. Second, comprehensive comparisons with multiple triage methods (commercial methylation kits, cytology, HPV16/18 testing, and p16/Ki-67 dual staining) offer robust clinical benchmarking data. However, some limitations should be acknowledged. The single-center design at Nanjing Drum Tower Hospital may affect generalizability to other populations. While real-time methylation-specific PCR provides sensitive detection, its quantification thresholds might influence low-level methylation assessments. Additionally, the cost-effectiveness of this six-gene test in routine screening requires further evaluation through health economic studies. These limitations highlight directions for future multicenter validation and implementation research.

Conclusions

In conclusion, our study demonstrates that the six-gene methylation test shows superior triage accuracy for CIN2+ and CIN3+, with methylation levels strongly correlating with disease severity. This Chinese-developed molecular test represents a promising advancement in cervical cancer screening, potentially reducing unnecessary colposcopies in HPV-positive women. The multi-gene approach offers improved specificity over conventional methods, addressing a critical need in clinical practice. Future multicenter validation studies and cost-effectiveness analyses are warranted to confirm its clinical utility before

widespread implementation. This test could significantly enhance cervical cancer prevention strategies, particularly in high-burden settings like China

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Conflict of interests

The authors declare no competing interests.

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