

Effective methylation triage of a screening population with HPV infection and/or abnormal cytology results in an Asian country

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Abstract

Background: The S5-methylation test, as an alternative classifier to cytology and HPV16/18 genotyping to triage cervical squamous intraepithelial lesions, has not been widely validated in Asian countries. Herein, we compared S5 methylation to HPV16/18 and cytology to detect cervical high-grade squamous intraepithelial lesion (HSIL) in a screening population who with either HPV infection or abnormal cytology results or both of them, derived from a multi-central clinical trial of 2246 Chinese participants. We matched all [?]¹HSIL+ cases (n=468) with [?]¹LSIL controls (n=468) to analyze the effectiveness of methylation. Methylation of S5 was quantified by pyrosequencing, blinded to cytology, histological and initial HPV results. **Results:** The S5 methylation could distinguish women with [?]¹HSIL+ from women with [?]¹LSIL at a high area under the curve (AUC) of 0.80 (95% CI 0.74-0.85). The sensitivity of S5 methylation (at 2.85 cutoff) for [?]¹HSIL+ was 76.1% (95% confidence interval [CI] 71.7-79.2) was higher than HPV16/18 sensitivity (64.9%, 95% CI 58.3-71.7, $P = 0.039$) or cytology (48.9%, 95% CI 42.8-53.2, $P < 0.001$). At this cutoff, the specificity of S5 for [?]¹HSIL+ was (79.9%, 95% CI 76.2-84.9), higher than HPV16/18 (44.8%, 95% CI 40.1-49.4, $P < 0.001$) and cytology (54.6%, 95%CI 50.7-57.9, $P < 0.001$). In addition, S5 methylation could provide predictive information about progression in specific population in follow-up period. **Conclusion:** S5 methylation classifier with high sensitivity and specificity exceeded HPV16/18 or cytology for detecting women with [?]¹HSIL+ in a screening Chinese population with HPV infection and/or abnormal cytology results. Furthermore, S5 methylation is a potential classifier for predicting progression.

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Abstract

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Results: The S5 methylation could distinguish women with [?]**HSIL+** from women with [?]**LSIL** at a high area under the curve (AUC) of 0.80 (95% CI 0.74-0.85). The sensitivity of S5 methylation (at 2.85 cutoff) for [?]**HSIL+** was 76.1% (95% confidence interval [CI] 71.7-79.2) was higher than HPV16/18 sensitivity (64.9%, 95% CI 58.3-71.7, $P = 0.039$) or cytology (48.9%, 95% CI 42.8-53.2, $P < 0.001$). At this cutoff, the specificity of S5 for [?]**HSIL+** was (79.9%, 95% CI 76.2-84.9), higher than HPV16/18 (44.8%, 95% CI 40.1-49.4, $P < 0.001$) and cytology (54.6%, 95%CI 50.7-57.9, $P < 0.001$). In addition, S5 methylation could provide predictive information about progression in specific population in follow-up period.

Conclusion: S5 methylation classifier with high sensitivity and specificity exceeded HPV16/18 or cytology for detecting women with [?]**HSIL+** in a screening Chinese population with HPV infection and/or abnormal cytology results. Furthermore, S5 methylation is a potential classifier for predicting progression.

Keywords: Cervical cancer; DNA methylation; S5; triage; progression; Biomarkers

Background

Uterine cervical cancer is one of the most leading causes of cancer death among women worldwide, especially among women in developing countries^{1,2}. According to the updated data from International Agency for Research on Cancer (IARC)³, it is estimated that of all the 604,127 new cases and 341,831 deaths^{4,5} of cervical cancer worldwide in 2020, and more than 1/6 of those cases occurred in China⁶. It was well acknowledged that persistent infection with high-risk human papillomavirus (hrHPV) is the cause of almost all cervical cancer⁷. However, only a small proportion of hrHPV infections persist⁸ and develop into cervical squamous intraepithelial lesion (SIL) which may, if left untreated, progress to cancer.

In 2019, the World Health Organization issued a call for action to eliminate cervical cancer⁹, and then advocated a series of approaches includes increasing HPV vaccine coverage¹⁰, increasing screening of women aged more than 30 years with hrHPV testing and treatment for hrHPV-positive (hrHPV+) women that are suspicious of cervical cancer precursor lesions. However, great discrepancies exist in the popularity and quality of screening methods. A combination of hrHPV+ DNA testing and cytology detection has been implemented as a routine classifier for cervical cancer screening in many countries, there are still several limitations including that cytology is subjective and requirement for pathologists, and hrHPV test with low specificity and might result in high colposcopy referral⁸. Although colposcopy is helpful to detect the cervical lesion, most HPV infections will not give rise to (pre)malignant disease, increased unnecessary colposcopy referrals would lead to unnecessary treatment and further would negatively affect childbearing^{11,12}. Thus, an adequate screening classifier is urgently needed for women with abnormal HPV and cytology results¹³.

An objective triage strategy which could be automated and incorporated molecular test combining with HPV detection might be able to solve these issues. As a primary form of epigenetic inheritance¹⁴, DNA methylation^{15,16} has been extensively studied and widely used for tumor classification^{17,18}, early detection¹⁹, therapy target, and predictive biomarker^{20,21} of metastasis and recurrence²². What's more, methylation assays can be automated, have accurate quantitation, are robust to operator variations and can be performed in the same specimen as the HPV testing. Of the more than 100 human methylation biomarker^{23,24} genes detected so far in cervical tissue¹³, several biomarkers have been repeated shown to have elevated methylation in cervical cancers and high-grade squamous intraepithelial lesion, which including ZNF671¹⁷, TERT²⁰, SOX1²⁰, CADM1²⁵, MAL²⁵, FAM19A4²⁶, miR-124-2²⁷, PAX1²⁸, JAM3²⁹, and EPB41L3³⁰.

The S5 methylation classifier is a test based on DNA methylation of the late regions L1 and L2 of HPV16, HPV18, HPV31 and HPV33 combined with the promoter region of human tumor suppressor gene EPB41L3³⁰ that identifies women with HSIL or more worse lesions. Here, S5 was developed in a colposcopy study, and well tested in the UK, Canada, and Mexico.

Although earlier studies in developed countries have shown that the performance of the S5 classifier to detect precancer lesion, there are few studies validating S5 in developing countries and such low and middle incomes settings. In this study, we figured out the efficiency and potential of S5 methylation in a screening population in China, including the 2246 women with abnormal results between HPV and cytology (2246 women who had any abnormal results for HPV and cytology). We compared the ability of the S5 methylation, repeated conventional cytology and HPV16/18 genotyping in detecting HSIL+ cases at the timepoint of 6 months, 12 months, 18 months and 24 months within the 2-year endpoint, among women with abnormal results between HPV and cytology selected from a multi-center study (the study recruited women from routine opportunistic screening services of China, a middle-income country) and attempted to find the evidence to support S5 as an optimal triage classifier.

Results

In the present study, we analyzed the effective S5 methylation in an Asian screening population from a multi-central clinical trial.

Characteristics of study population

All the 7033 finished sample collection for HPV, cytology, and methylation testing in this study. **Figure 1** shows that 2246 of 7033 women included in the study, and that 602 (24.4%) in the IC (immediate colposcopy) group, 1644 (66.7%) in the RF (repeat follow-up) group. Among the 2246 women with either abnormal HPV results or abnormal cytology results or both abnormal results, 1414 (57.34%) had an adequate histological diagnosis, 943 of which had a diagnosis of [?]LSIL (411 NEG and 532 LSIL) and 471 of [?]HSIL+ (401 HSIL and 70 cancers). After exclusion of 4 samples (2 cases inadequate in sample materials and corresponding controls), 41.8% (172/411), 55.8% (297/532), 99.5% (399/401) and 100% (70/70) of women with negative, LSIL, HSIL or CC histopathological diagnosis were included in this analysis.

Table 1 describes the characteristics of the included 468 [?]HSIL+ cases and 468 [?]LSIL controls. Cases and controls had similar age, time to diagnosis, marital status, number of sexual partners, smoking or not and number of sexual partners. HPV16 was confirmed in 362 (38.6%), HPV18 in 279 (29.8%), HPV31 in 127 (13.5%) and HPV33 in 60 (6.4%) of all samples. The sensitivity and specificity of S5 is different among IC cohort and RF cohort (Table 2). In addition, the sensitivity (72.9%, 95% CI 70.7-75.1) of S5 methylation in RF cohort was better than the performance of HPV16/18 (66.3%, 95% CI 62.1-70.3, $P = 0.002$) and cytology (48.2, 95% CI 42.7-54.1, $P = 0.008$) in the RF cohort.

S5 methylation and EPB41L3 were related to the severity of SIL

Median S5 methylation (**Figure 2**) increased proportionally (Cuzick test for trend was significant, $P < 0.001$) with histological diagnosis: 1.31 in histology Negative (n=172), 2.34 in LSIL (n=297), 12.7 in HSIL (n=399), 18.8 in cancer (n=70). **Figure 3** shows similar pattern of methylation levels increasing by lesion severity for EPB41L3 (Cuzick test for trend was significant, $P < 0.001$).

Furthermore, to assess if there was a significant difference between HPV16/18-negative and HPV16/18-positive women stratified by SIL status, we consider the methylation of human gene EPB41L3 in all samples. **Figure 4** shows that there was very no significant difference between the < HSIL+ HPV16/18-positive and the < HSIL+ HPV16/18-negative samples ($P = 0.14$).

Performance of the S5 classifier to detect [?]HSIL+ cases

ROC analysis of the S5 methylation for detecting [?]HSIL+ cases is shown in **Figure 5**. The cutoff value of 2.85 was selected for discrimination of "[?]HSIL+" lesions from "[?]LSIL" diagnoses in this present Chinese screening population, with the best AUC value as 0.80. Also, we validated the performance of

S5 methylation with different cutoff value, in the present population (cutoff = 2.85) and in other specific populations (S5 cutoff 0.8 in a UK colposcopy referral population as previous reported³¹). The receiver operation characteristic curve (ROC) analysis demonstrated that the S5 methylation had an AUC (area under the curve) of 0.80 (95% CI 0.74–0.85) for detecting HSIL+ among abnormal results between HPV and cytology (**Figure 5**), which was similar to the performance in a China colposcopy referral population¹³ (ACU = 0.86) as previous reported. Also, we calculated the AUC of EPB41L3 (0.65, 95% CI 0.74–0.85), which was much lower than that of S5 methylation (0.80, 95% CI 0.74–0.85) (**Figure 6**). According to the area under the curve (AUC), when combining sensitivity and specificity, the accuracy of S5 at a cutoff 2.85 for detection of HSIL+ was significantly higher than the accuracy of the HPV test or combination HPV with cytology (**Figure 5**).

Cross tabulation of the number of cases or controls with negative or positive test results and the comparison of sensitivity and specificities of the tests to detect [?](His)HSIL+ are shown in Table 3 and 4, respectively. For detecting [?](His)HSIL+ cases, the sensitivity of HPV16/18 was 64.9% (95% CI 58.3–71.7) and of cytology was 48.9% (95% CI 42.8–53.2) (as most cases with LSIL or HSIL were HPV infected, here we analyzed the performance of HPV16/18 genotyping). S5 at the cut-point of 0.8 (predefined in a UK population) had a higher sensitivity (95.5%, 95% CI 92.6–97.1) but significantly lower specificity (19.4%, 95% CI 17.8–21.7) than HPV 16/18 (44.8%, 95% CI 40.1–49.4, $P < 0.001$) or cytology testing (54.6%, 95% CI 50.7–57.9, $P < 0.001$). At the cut-point of 2.85 (corresponds to setting the specificity of S5 for [?](His)LSIL at which is higher than cytology specificity), the S5 specificity was significantly higher than HPV16/18 genotyping ($P < 0.001$), while the sensitivity of S5 was 76.1% (95% CI 71.7–79.2), which remained significantly higher than sensitivity of HPV16/18 genotyping, cytology. When compared the performance of S5 methylation with the scenario “cytology plus HPV16/18” (scenario positive means that positive for any of the two tests), the sensitivity and specificity of S5 were also better than the scenario “cytology plus HPV16/18” (Table 4).

S5 classifier in predicting progression to [?](His)HSIL+

Figure 7 shows a significant difference (Likelihood-ratio test, $P = 0.02$) between cumulative proportions of progression to [?](His)HSIL+ distributed by time in women population: (“HPV positive except HPV16/18” and “abnormal cytology results except [?](TCT)HSIL”). In this specific population, comparison between women positive for the S5 methylation classifier (S5 methylation classifier [?] 2.85) vs negative for the S5 methylation classifier (S5 methylation classifier < 2.85) were conducted. As depicted in **Figure 7**, most of the cases (“HPV positive except HPV16/18” and “abnormal cytology results except [?](TCT)HSIL”) who progressed to [?](His)HSIL+ within 2 years, were calculated S5 methylation scores equal or greater than 2.85 ([?] 2.85) at the first visiting.

Discussion

This is the first study to assess the predictive potential of the S5 methylation classifier in a larger screening setting of patients with abnormal results between HPV and cytology in an Asian country. S5 classifier, a multigene methylation test, which has shown good performance in the United Kingdom^{30,32}, Canada³³, and Mexico³¹, Colombia³⁴ and recently in a China colposcopy referral population conducted by our team¹³. In this study, we enrolled the screening population as study setting, and we evaluated the predictive potential of S5 methylation for progression of specific untreated squamous intraepithelial lesion.

Here, we validated the effectiveness of S5 classifier at the cutoff 2.85 in the present screening population setting. We validated a DNA methylation classifier of HSIL+ histology, among women with abnormal results between HPV and cytology from a China screening population. Our study included 938 baseline samples (including HPV, cytology and S5 methylation) from women who were referred colposcopy at first screening or had 2 years of active follow-up and culminating in colposcopy clinics directed biopsy diagnoses if positive for either HPV or cytology tests. To address potential bias in methylation levels by age at diagnosis or time of follow-up, our controls were age and time to diagnosis matched and randomly chosen among all baseline [?](His)HSIL+ women. We conducted an independent analysis in the RF arm and IC arm in Table 2, although the sensitivity and specificity of S5 is different, the result is still in line with the overall conclusion

(which means that the sensitivity and specificity of S5 in both cohorts are better than the performance of HPV16/18 and cytology). The genotyping and methylation assays, as well as the verification of the histological diagnoses, were conducted independently and blindly after the end of the study. To date, there are few reports comparing the performance of cytology and HPV16/18 genotyping with DNA methylation assays to detect HSIL+ in women with abnormal results between HPV and cytology. The study compared the performance of S5 for HSIL+ detection to repeatedly cytology testing and HPV genotyping in women with abnormal results between HPV and cytology in developing countries setting, objectively.

The AUC obtained in this study was 0.80 (95% CI 0.74-0.85). At a specificity set at 79.9% that corresponds to using S5 at a cut-off value of 2.85, the sensitivity of S5 for HSIL+ were 76.1%, which were significantly higher than the sensitivities of HPV16/18 ($P = 0.039$) or cytology ($P < 0.001$). In addition, S5 methylation classifier also outperformed the comparator triage testes (HPV and cytology) and their combination regarding the specificity. Considering that we are comparing different triage tests among women with above the average risk for HSIL+ (cytology[?](TCT)HSIL or/and hrHPV+), S5 acts as a good triage alternative since this test decreased the false positive rate by near 35% (Table 4) and exhibited higher sensitivity than HPV 16/18, cytology, or combination of these two tests for both HSIL+ endpoints. This characteristic of S5 is especially valuable for remote areas of developing countries, where higher sensitivity is crucial to identify at-risk women in fewer screening visits and decreasing the use of resources to follow-up women with low risk of disease.

In our study, cytology had a specificity of 54.4%, but had the test with by far the highest false negative rate. We recognized that the sensitivity of our cytology was lower than the performance of cytology in several studies in developed countries, but several studies have demonstrated that the sensitivity in developing countries may be as low as 30%. Although the selection bias about population occurs, it is worth noting that despite many efforts to improve cytology quality and sensitivity, difficulties in quality controlling and delays in diagnosis still prevail. We used the predefined cutoff at UK population (S5 cutoff = 0.8) that corresponded to previous studies with S5^{31,32}, while the S5 at cutoff 0.8 (95.5%) vs the sensitivity of S5 at cutoff 2.85 (76.1%), the specificity(19.4%, 95% CI 17.8-21.7) at 0.8 cutoff is much lower than 79.9% at 2.85 cutoff. Future work is planned to assess the performance of S5 as a triage to determine appropriate cutoffs within a screening population. Also, further work is needed to determine the performance of the S5 methylation assay in vaccinated populations.

Also, we analyzed the distribution of S5 panels scoring and host-cell gene named EPB41L3 scoring among a screening population (**Figure 2 and Figure 3**), the results were in line with the distribution among the colposcopy referral population. Additionally, the changes in the level of methylation of S5 panels and host-cell gene named EPB41L3 compared if methylation of EPB41L3 was different among different cytology results and different HPV status, the result showed very little difference ($P = 0.31$) (**Figure 4**). This result indicates that it is difficult to conduct a screening test only by measuring the methylation levels EPB41L3, which was in line with some other previous studies^{29,32}.

What's more, we reveal a new utility of the S5 DNA methylation classifier measurement, specifically as a classifier not only for an enlarged screening population, but also for assessing risk of progression specific untreated squamous intraepithelial lesions. A promising prognostic test for SIL could greatly improve the current screening and even alter further treatment algorithms. A generally acknowledged difficulty of current strategies lies in the subjectivity and inter-observer variability in not only cytological but also histological diagnoses. These limitations result in misclassification of lesions, unnecessary colposcopy referrals, multiple follow-up visits, and either delayed treatment or over-treatment, exacerbating the potential harm to the patient meanwhile excessive burdening to medical practice. An improved predictive test could optimize the current management so that cases with progressive potential could be treated sooner and cases with regressive potential perhaps be indicated untreated for longer periods to allow more regressions. It should be considered that the natural history of long-term HPV persistence with respect to eventual true progression of abnormal screening results (abnormal screening results between HPV and cytology) to [?]HSIL+ beyond 2 years remains unclear. According to the current Chinese clinical algorithm, persistence of abnormal screening results (abnormal screening results between HPV and cytology) for 2 years was an indication for colposcopy

referrals or even further treatment; however, we do not know what proportion of these colposcopy referrals were necessary to prevent cervical cancer.

In this study, of all the women (abnormal results between HPV and cytology; meanwhile HPV16/18- and < (TCT)HSIL result of cytology) progressed to [?](TCT)HSIL+ within 2 years, most of these cases were positive for the S5 methylation classifier (S5 methylation classifier [?] 2.85) at the first visiting. All the cases with HPV16/18 positive or cytology [?] (TCT)HSIL+ were referred to immediate colposcopy according to the current Chinese algorithms, thus it is not clear the proportion of progressed women with HPV16/18 positive or cytology [?] (TCT)HSIL+, and it is plausible that further prospective study is needed to demonstrate the issue. Even so, these results fully indicate that S5 methylation classifier with great potential to predict the progression of women with abnormal results between HPV and cytology.

According to the call for “action to eliminate cervical cancer” issued by the World Health Organization in 2019⁹, increasing effective screening of women with hrHPV testing is necessary. However, it is difficult to implement the HPV DNA testing frequently for populations living in remote areas where there few opportunities to screen women at proper intervals and for follow-up after screening^{4,35}. What’s more, most HPV infections are transient so immediate ablative treatments can lead to unnecessary gynecological harms for women with low risk of disease³⁶. For this reason, primary screening with HPV testing and/or cytology requires other triage tests to identify women at high-risk of high-grade disease among those who are with abnormal results between HPV and cytology. Considering recalling women for a second test after screening is challenging or impossible in developing countries; therefore, risk triage should ideally occur at the screening visit in these settings. Hence, these settings have unique needs for triage strategies.

Considering these results, it is possible to envisage a screening test that simultaneously the current tests (HPV genotyping and cytology) and measurement of S5 methylation. As depicted in **Figure 8**, such potential screening strategy would provide the benefit of immediate and more accurate results for triaging women as below: (I) negative for all biomarkers, who would go back to routine screening; (II) referred to colposcopy (according to the current algorithm: HPV16/18 positive or cytology [?] (TCT)HSIL+ or repeated abnormal result among HPV and cytology) and methylation positive, would be given priority for colposcopy inspection than who referred colposcopy and methylation negative, and (III) repeated follow up (according to the current algorithm) and methylation positive, would be given priority for follow-up at proper intervals than who were repeated follow up and methylation negative, in developing countries especially some remote areas.

The strength of this study is the validation of the S5 classifier in a routine screening study in the China with blinding of all results to the lab technicians, and the use of prespecified cutoffs for the methylation classifiers which minimized the risks for bias and overfitting. In practice, hrHPV-positive women could have the methylation tests performed on the original samples in a reflex manner, triaging women at risk to colposcopy and thereby reducing anxiety and overtreatment in the low-risk women. Possible concerns over missing some of the LSIL and HSIL might be addressed by referring women negative or low risk by the DNA methylation classifier to repeat HPV testing and cytology in proper follow-up duration.

A weakness is that our study was restricted to the length of follow-up short, which may result in some bias. While potential statistical bias, it is under the circumstance of the current algorithm in China based on the guideline: all the women (with HPV16/18 positive and/or cytology results [?](TCT)HSIL+) were advised to refer colposcopy. Thus, it is difficult to arranged women for immediate colposcopy or follow up randomly, also difficult for RF cohort undergo colposcopy before the following-up. Considering that in this study we focused the screening population (with abnormal screening results) at the first visiting, IR cohort and RF cohort were both included. Since the RF cohort including women with (“HPV positive except HPV16/18” and “abnormal cytology results except [?](TCT)HSIL”), who were followed-up every 6 months for 2 years follow-up period, and need 2 years following up period to determine whether to be enrolled in, the current study (including IC cohort and RF cohort) lasted for a long time to complete the data collection. However, a larger population is needed in the further study. In addition, our results might not be directly generalized to other histological diagnosis (diagnosis using cervical intraepithelial neoplasia in some studies) properly.

Despite the differences in study designs and in the proportion of the HSIL+ cases be included, our results are remarkably similar to other studies^{30,32,33}. The S5 methylation test accurately identified women with higher risk of cervical high-grade disease and cancer among those who with abnormal result between HPV and cytology. Even further, our study demonstrated that S5 outperforms both cytology and HPV16/18 in detecting HSIL+ in Chinese women. Currently, S5 DNA methylation test is labor intensive and costly. Based on the practice of our study¹³, the cost of an S5 test is not higher than that of a routine HPV screening and reflex genotyping, and it could be balanced against the expected 30 to 50% reduction in colposcopy referral costs. The recent developments of affordable and scalable next-generation sequencing assays strengthen our proposal that the S5 DNA methylation classifier test may be an acceptable strategy for the triage of women with abnormal result between HPV and cytology in developing countries. Strategies combing screening and immediate clinical management are urgently needed for developing countries settings where infrastructure for follow-up after screening is limited. S5 might be a potential classifier in developing countries for reducing costs and encouraging doctors to focus on the women at real risk of cervical cancer. Our results warrant further clinical validation of S5 in larger prospective population-based screening trials.

Conclusion

S5 methylation had better sensitivity and fewer false positives than cytology plus HV16/18 to predict cervical high-grade squamous intraepithelial lesion or worse ([?]HSIL+) in a selected cohort (who with abnormal cervical screening results) in an Asian country. Additionally, S5 methylation is potential for providing information to predict progression. Moreover, S5 is a potential classifier in LMICs as affordable methylation tests become available.

Methods

Ethical approval

This study was approved by the Obstetrics and Gynecology Hospital of Fudan University Institutional Review Board, and the study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. Oral and written informed consents were obtained from the patients or their guardians before the study, and those who agreed were administered a survey to collect demographic data.

Study population

Participants were women who visited gynecology clinics in four hospitals for routine cervical cancer or precancer screening between December 2017 and November 2018, aged from 27 to 70 years. The protocol, inclusion and exclusion criteria of the study are shown in **Figure 1**.

At the first visiting for gynecological examination in multi-center clinics, cervical samples were obtained to conduct the HPV detection, cytology and S5 methylation analyses. Women with HPV-positive results and/or abnormal cytology results (HPV positive and/or cytology with ASCUS, LSIL, ASC-H, HSIL or CC) at the first visiting time were recruited in the study. All the included participants were divided into two groups, women with HPV16/18+ and/or [?] (TCT)HSIL result of cytology at first visiting time were divided into receive immediate colposcopy group (IC group; n=602), women with (“HPV positive except HPV16/18” and “abnormal cytology results except [?] (TCT)HSIL”) were divided into repeat follow-up group (RF group; n=1644) including scheduled cytology and HPV tests every 6 months for 2 years follow-up period (which means scheduled study visit at 6, 12, 18 and 24 months). Colposcopy and biopsies were performed if women were divided into the IC group, also colposcopy and biopsies were performed if either repeated [?]LSIL or repeated hrHPV+ was observed during the 24-month follow-up period in the RF group. All the histological samples and cytology samples were reviewed by a standardized group of pathologists, all the pathologists and cytopathologists were blinded to the HPV infection status.

Selection of methylation sub-group participants and study protocol

Cases were women identified after the end of multi-center study as women who had abnormal screening

results at baseline (first visiting) and with a colposcopy-directed biopsy diagnosis of (His)HSIL or worse (including (His)HSIL, carcinoma in situ, and cervical cancer (CC)) at any time during the 2-year follow-up. Controls were randomly selected regardless of arm allocation from women who were abnormal screening results at baseline, had a biopsy with a diagnosis of less than (His)LSIL during the follow-up confirming that they were at low risk of cervical cancer and with enough remainder of archived baseline samples in specimen transport medium for further testing. Controls were individually matched to cases by age. As shown in the flowchart in **Figure 1**, the 471 cases and 471 matched controls were identified for the study. Briefly, we enrolled samples (with initially an HPV positive or abnormal cytology result) derived from IC cohort and RF cohort, and then grouped as “cases group (with histology [?]HSIL+)” and age-matched “control group (with histology [?]LSIL)”.

In this study, according to “The Lower Anogenital Squamous Terminology Standardization Project for HPV-associated Lesions” (LAST), we categorized histopathological results as “No-Lesion”, “LSIL”, “HSIL” and “CC”. And we categorized all the cytology results as “NILM”, “(TCT)LSIL”, “(TCT)HSIL” and “CC”, “ASCUS” was grouped in “(TCT)LSIL”, and “ASC-H” was grouped in “(TCT)HSIL”. Based on the histopathological diagnoses as “golden standard”, we compared the diagnostic performance of S5 methylation with other existing classifier among women with abnormal results with HPV or cytology.

All the cervical samples were obtained from the first visiting in multi-center clinics. Samples for cytology were collected using Cervi-Brush® bush (Rovers® Medical Devices, North Brabant, Netherlands) and stored in a Sure-Path vial for further cytology analysis, and samples for HPV and methylation detection was also collected and then stored at 2°C to 8°C. The vials used for testing were stored 2°C to 8°C upon arrival the central cytology laboratory of Gy&Ob hospital of Fudan University in Shanghai, China.

Genotyping of HPV

HPV genotyping was performed with Type-specific HPV genotyping kit (Bioperfectus Ltd, Jiangsu, P.R. China). After vials for HPV genotyping were transported to the HPV laboratory of Gy&Ob hospital of Fudan University, a qualitative in vitro assay was performed to detect the result for HPV16, HPV18 and other 12 HPV genotypes, including HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

S5 DNA Methylation testing

Methylation assays were based on end-point PCR and quantitative pyrosequencing of amplicons using primers for late regions L1 and L2 of HPV16, HPV18, HPV31 and HPV33 combined with the promoter region of human tumor suppressor gene EPB41L3. Briefly, genomic DNA was extracted using 100 µL of suspensions with a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) and DNA was quantified by UV absorption. 250 ng of DNA was used in the bisulfite treatment where un-methylated cytosines were converted to uracil with the EpiTect Fast Bisulfite Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Converted DNA was eluted in 15 µL Buffer EB twice and the eluant was combined for next steps. Converted DNA (2µL/sample) was added to the PCR mix system with 12.5 µL of 2× PyroMark PCR master mix (Qiagen) and optimized concentrations of primers and Magnesium chloride. Polymerase chain reaction was conducted on a Bioer Technology LifeECO amplification instrument (TC-96/G/H(b)C model, Hangzhou Bioer Technology Co., Ltd.). The PCR products were used for the followed pyrosequencing (PyroMarkQ48 Autoprep, Qigen, Hilden, Germany). The methodological details were detailed in several studies¹³. The laboratory was blinded to HPV genotyping results before the specimen was performed S5 methylation assay to minimize these unintentional biases.

Statistics

Demographic, and sexual behavior of participants were summarized as means or proportions. Our primary aim was to evaluate the clinical diagnostic performance of the S5 methylation classifier. The standardized equation for S5 scoring was used to calculate the average methylation values of the five target regions:

$$S5 = EPB41L3*(30.9) + HPV16L1.3*(13.7) + HPV16L2*(4.3) + HPV18L2*(8.4) + HPV31L1*(22.4) + HPV33L2*(20.3)$$

We created boxplots to illustrate the distribution of the S5 classifier histopathological diagnosis of the lesions (No-lesion, LSIL, HSIL and cervical cancer (CC)). We used the Mann-Whitney U test for comparing S5 scoring differences between different categories and the Cuzick test for trend to determine if methylation increase significantly as a function of great histopathology result.

To maximize the sensitivity and specificity of the S5 score after the triage test (HPV16/18 and cytology), we created receiver operating characteristic (ROC) curve to estimate areas under the curve (AUC), and we validated an effective S5 cutoff value from several S5cutoff values to discriminate [?] (His)HSIL+ cases from < (His)HSIL+ cases via the post-hoc analysis. Another analysis was done using the establishment 0.8 cutoff validated in UK screening population^{30,32} for detection of [?] (His)HSIL+.

We compared the sensitivity and specificity as the disease endpoint of [?]HSIL+, and compared with the other tests such as cytology and HPV detection, which are the currently recommended triage tests for women in the Chinese screening guidelines.

A ROC curve was also computed for compare the performance of S5 methylation and EPB41L3.

All p values were estimated as two sided, with a confidence interval of 95%. Statistical analyses were performed by SPSS statistical software version 25.0 (IBM, NY) and Excel. $p < 0.05$ was considered significant statistically.

Abbreviations

ASCUS: atypical squamous cells of undetermined significance; ASC-H: Atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion; NILM: No intraepithelial lesion or malignancy; ROC: Receiver operating characteristic; SIL: Squamous intraepithelial lesion; CPG: Cytosine-phosphate-Guanine site; hrHPV: High-risk human papillomavirus; No-lesion: histologically negative without squamous intraepithelial lesion; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CC: cervical cancer; TCT: Thinprep cytologic test; (His)HSIL: high-grade squamous intraepithelial lesion for histological diagnosis; (TCT)HSIL+: cytological high-grade squamous intraepithelial lesion or worse for TCT result; L1: Late region 1; L2: Late region 2; LMIC: low- and middle-income country; PPV: Positive predictive value; NPV: negative predictive value; IC group: immediate colposcopy group; RF group: repeat follow-up group.

Authors' contributions

Guan Nan Zhou: data management and analysis, draft manuscript. Yuan Yuan Gu: extract raw data, draft manuscript. Qing Wang: review medical cord and data extraction at Department of Gynecology, the Obstetrics and Gynecology Hospital of Fudan University, read and edit manuscript. Ke Qin Hua: review medical cord and data extraction at Department of Gynecology, the Obstetrics and Gynecology Hospital of Fudan University, read and edit manuscript. Jing Xin Ding: review medical cord and data extraction at Department of Gynecology, the Obstetrics and Gynecology Hospital of Fudan University, read and edit manuscript.

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Availability of data and materials

The full datasets are not publicly available due to the need to protect participant confidentiality; however, the data that support the findings of this study are available on request from the corresponding author.

Inquires should be communicated to corresponding author who will consider all sufficiently specified and reasonable requests.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of our hospitals, and the study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. Oral and written informed consent were obtained from the patients or their guardians before the study.

Competing interests

All the authors declare no competing interests.

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Figure Legends:

Figure 1. Flowchart of the study showing the numbers of women in each step. All the included participants with abnormal results between HPV and cytology were divided into two groups, women with HPV16/18+ and/or [?](TCT)HSIL result of cytology at first visiting time were divided into receive immediate colposcopy group (IC group; n=602), women with (“abnormal results between HPV positive except and cytology; meanwhile HPV16/18-” and “abnormal cytology results except [?](TCT)HSIL” result of cytology) were divided into repeat follow-up group (RF group; n=1644).

Figure 2. Boxplot distribution of S5 score according to the histological diagnosis: NEG, LSIL, HSIL, and cervical cancer (CC). The median and interquartile ranges are depicted by boxes. Cuzick test for trend was significant ($P < 0.001$). The upper whisker extends to the largest point of the inter-quartile range from the upper quartile. The lower whisker extends to the smallest point of the inter-quartile range from the lower quartile.

Abbreviation: NEG, histologically negative without squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; CC, cervical cancer.

Figure 3. Boxplot distribution of EPB41L3 score according to the histological diagnosis: NEG, LSIL, HSIL, and cervical cancer (CC). The median and interquartile ranges are depicted by boxes. Cuzick test for trend was significant ($P < 0.001$). In the box plot, it contains six data expressions. Arrange a set of data from the maximum (upper edge), upper quartile Q3, median (darkest black line), lower quartile Q1, minimum (lower edge), and outlier. The upper whisker extends to the largest point of the inter-quartile range from the upper quartile. The lower whisker extends to the smallest point of the inter-quartile range from the lower quartile. Abbreviation: NEG, histologically negative without squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; CC, cervical cancer.

Figure 4. Percentage distribution of EPB41L3 methylation by hrHPV positive and SIL status in patients with abnormal results between HPV and cytology. Of particular interest was a methylation cut-off point for

EPB41L3 when a sample was not positive for HPV 16, 18, 31 or 33; or unmethylated if positive, which was simple calculated at the predefined cutoff for S5 classifier as $S5 = 2.85/30.9$ [?] 9.2%.

Figure 5. Receiver operating characteristic (ROC) of S5 classifier for detecting HSIL+. The red circle denotes the sensitivity and specificity of S5 at cutoff of 0.8 for HSIL+. The yellow triangle denotes the sensitivity and specificity of S5 at cutoff of 3.7 for HSIL+. Cytology (first [?] HSIL result of cytology, or [?] HSIL result of cytology at 6 or 12 months after first time), HPV16/18 and cytology plus HPV16/18 are depicted. In the ROC (receiver operator characteristic curve), AUC (Area Under Curve) is defined as the area under the ROC curve enclosed by the coordinate axis, the larger the AUC, the better the credibility. The y-coordinate value of yellow triangle indicates the sensitivity of S5 at 2.85 cutoff, is 0.761. The x-coordinate value of the yellow triangle indicates the (1-specificity) of S5 at 2.85 cutoff, is 0.201, which means the specificity of S5 at 2.85 cutoff is 0.799. HSIL+, high-grade squamous intraepithelial lesion or worse; (TCT)HSIL+, cytological high-grade squamous intraepithelial lesion or worse for TCT result.

Figure 6. Receiver operating characteristic (ROC) curves of S5 and EPB41L3 methylation site for detecting HSIL+. The blue curve denotes the S5 for detecting HSIL+. The red curve denotes the EPB41L3 for HSIL+. HSIL+, (histological) high-grade squamous intraepithelial lesion or worse.

Figure 7. Cumulative proportions of women who progressed to [?](his)HSIL+ by time since the first visiting with “HPV positive except HPV16/18” and “abnormal cytology results except [?](TCT)HSIL”. In this analysis, <(his)HSIL although following-up repeated (“HPV positive except HPV16/18” and “abnormal cytology results except [?](TCT)HSIL”) were regarded as non-progressions. Among this population, the graph shows the distribution by time (in months) of women S5[?]2.85 (solid line) vs S5 classifier <2.85 (dashed line). There was a significant difference between these predictors (Likelihood-ratio [LR] test $P = 0.02$).

Figure 8. Potential modified screening strategy for triaging women with abnormal screening results. Firstly, for negative for HPV and/or cytology population, giving priority to routine screening for S5 positive women. Secondly, for women with HPV16/18 positive or cytology [?] (TCT)HSIL+ or repeated abnormal result among HPV and cytology, giving priority to colposcopy inspection for S5 positive women. In addition, for women with “HPV positive except HPV16/18” and/or “abnormal cytology results except [?](TCT)HSIL”, giving priority to repeated following-up in more strict intervals for S5 positive women.



