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ORIGINAL RESEARCH

A Predictive Model Using Six Genes DNA Methylation Markers to Identify Individuals With High Risks of High-Grade Squamous Intraepithelial Lesions and Cervical Cancer

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Background: Cervical cancer is preceded by low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL). Human papillomavirus (HPV) test is a sensitive method for cervical cancer screening, but it is less specific compared with cytological examination, leading to overtreatment and reduced patient compliance. Therefore, new detection methods that can improve the accuracy of cervical cancer screening are needed.

Methods: In the present study, cervical exfoliated cell samples were collected from 228 Chinese individuals, including 114 healthy control individuals, 46 patients with LSIL, 21 patients with HSIL and 47 patients with cervical cancer. The DNA methylation levels of 12 cervical cancer-related genes were detected using quantitative multiplex methylation-specific PCR. All individuals were divided into high- or low-risk groups. Patients with HSIL and cervical cancer were assigned to the high-risk group, whereas healthy controls and patients with LSIL were assigned to the low-risk group. The ability to predict cancer risks was evaluated using ROC curves and a predictive model for cancer risk was constructed by linear regression analysis.

Results: The methylation levels were significantly higher for all 12 genes in individuals with cervical cancer or HSIL, compared with those in LSIL or normal group. Family with sequence similarity 19 member A4 (*FAM19A4*), phosphatase and actin regulator 3 (*PHACTR3*), somatostatin (*SST*), Zic family member 1 (*ZIC1*), paired box 1 (*PAX1*) and zinc finger protein 671 (*ZNF671*) were used to construct a predictive model for cancer risk prediction, with a specificity of 89.6% and a sensitivity of 95.0%.

Conclusion: The present study demonstrated the methylation levels of 12 cervical cancer-related genes were higher in Chinese patients with HSIL or cervical cancer. Also, a predictive model was constructed to distinguish cervical cancer or HSCL from individuals with low risk.

Keywords: cervical cancer, high-grade squamous intraepithelial lesions, methylation, predictive model, cancer risk

Introduction

Cervical cancer is the most common gynecologic malignancy and the fourth lethal cancer in women worldwide, accounting for 6.5% of new cancer cases each year in women.[1] In China, it is conservatively estimated that there are about 100,000 confirmed cases and about 30,000 deaths annually.[2] Cervical cancer is preceded by low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL).[3] According to the current

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guidelines for cervical cancer screening, abnormal test results from a Pap smear or Thinprep Cytologic Test (TCT) combined with HPV testing can lead to further invasive diagnostic procedures, such as colposcopy, as well as cervical biopsy is needed for histopathological diagnosis of HSIL, whereas clinical intervention is not required for LSIL, for which only timely follow-up is needed.[4,5] Thus, accurately identifying HSIL and more advanced lesions is an important goal of cervical lesion screening.

The cytological examination is the main method for cervical cancer screening, however, the diagnosis of atypical squamous cells of undetermined significance (ASCUS) is rarely included in a Pap smear screening and ASCUS is often not considered an abnormal category, leading that it has low sensitivity, and it is difficult to achieve high-quality cytopathological levels in developing countries, especially in remote areas.[6,7] A previous study including 176,464 individuals showed that even high-quality cytology tests had an omission diagnostic rate of at least 30% in HSIL and cervical cancers.[8] Compared with the cytological examination, the human papillomavirus (HPV) test is a more sensitive method, but its specificity is low.[9] HPV test increases the rate of clinical colposcopy referrals, while leading to frequent testing, invasive diagnostic procedures and overtreatment, ultimately leading to reduced patient compliance. New detection methods with high sensitivity and specificity in cervical cancer screening are needed.

Methylation, as an important form of epigenetics, plays a significant role in tumorigenesis and development.[10] DNA methylation patterns are strongly associated with cancer phenotypes, therefore they might be used in different aspects of cancer diagnostics.[11] At present, over 100 methylation markers have been detected in cervical tissues. Among them, about 20 genes have elevated methylation levels in HSIL or cervical cancer, including somatostatin (*SST*),[12] zinc finger protein 671 (*ZNF671*),[13] Ezrin,[14] paired box 1 (*PAX1*),[15] SRY-box transcription factor 1 (*SOX1*) and POU class 4 homeobox 3 (*POU4F3*).[16] Moreover, DNA methylation marker panels, such as GynTect had shown encouraging performance and higher specificity compared with the HPV-based cervical cancer screening.[17,18] However, few such studies were carried out in Chinese patients.[19]

The present study investigated the DNA methylation levels of 12 genes associated with the progression of cervical cancer in Chinese patients at different disease stages. In addition, the present study aimed to construct a model to distinguish individuals with cervical cancer and HSIL from LSIL and healthy women.

Materials and Methods

Samples Collection

Cervical cytology samples from a total of 228 women were collected from October 2021 to July 2022 at Shanghai First Maternity and Infant Hospital. These individuals were categorized into four groups: Normal (control; n = 114); LSIL (n = 46); HSIL (n = 21); and cervical cancer (n = 47).[20] TCT was used for the collection of samples, which is a basic screening method for cervical cancer, and would obtain a larger sample size than a blood sample, with higher sensitivity.[21] In brief, a 2-mL cervical cytology sample from each individual was collected using the TCT cervical brush. Women fulfilling the following criteria at the baseline visit were included in the study: (i) Age between 20 and 75 years; (ii) no previous HPV vaccination; (iii) no pregnancy; (iv) no previous cervical treatments for intraepithelial lesions; (v) no human immunodeficiency virus (HIV) infection or other cause of immunosuppression; (vi) the sample with negative for both TCT and HPV test, or no lesion for the histological diagnosis included in normal group; (vii) the biopsy sample with a histological diagnosis of LSIL/CIN grade1 included in LSIL group; (viii) the biopsy sample with a histological diagnosis of HSIL or CIN grade2/3 included in HSIL group; and (ix) patients diagnosed with cervical cancer according to National Comprehensive Cancer Network (NCCN) guidelines were included in the cervical cancer group.[22] The present study was approved by the Ethics Committee of Shanghai First Maternity and Infant Hospital (ethical approval no. KS21275) and conducted following the Helsinki Declaration.

DNA Isolation

DNA was isolated using the QIAamp DNA Mini kit (Cat. No. 51306, Qiagen GmbH) according to the manufacturer's instructions. DNA concentration was measured using Qubit (Thermo Fisher Scientific, Inc).

DNA Methylation Analysis Using Quantitative Multiplex Methylation-Specific PCR (qMSP)

DNA was bisulfite-converted using the DNA Methylation kit (Shanghai Yuanqi Bio Pharmaceutical Co., Ltd). For methylation analysis, Methylation-specific PCR Master Mix (Shanghai Yuanqi Bio Pharmaceutical Co., Ltd). was used, together with fluorescent dye-labeled Taqman probes, 50 ng of bisulfite-converted DNA and 100–300 nM of each primer. The specific primer sequences and Taqman probes used for 12 DNA methylation markers in promoter region (CpG islands) were designed by the researchers of this project (Table S1 in Supplementary Material). The following thermo-cycling conditions were used for the qPCR: Initial denaturation at 94°C for 5 min; followed by 50 cycles of 94°C for 15 sec and 60°C for 30s. ΔCq ratios were computed using the comparative Cq method, normalizing target Cq values respectively to ACTB.

HPV Testing

HPV testing was performed using the HPV GenoArray Test Kit (HybriBio Ltd., China). This assay could determine 21 hPV types, including HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, HPV53, HPV56, HPV58, HPV59, HPV66, HPV68 and HPV-CP8304, by the flow-through hybridization technique using HPV DNA amplified by PCR. In brief, the PCR reaction was set up by combining 1 μL of sample DNA with 23.25 μL of PCR premix and 0.75 μL of DNA Taq polymerase provided in the kit. The positive controls and negative controls from the kit were included in each PCR analysis process. The amplification was conducted under the following conditions: a denaturation at 95°C for 9 min; 40 cycles at 95°C for 20s, 55°C for 30s, and 72°C for 30s; finally, an extension at 72°C for 5 min. The flow-through hybridization was conducted on a preheated instrument at 45°C, using an HPV-21 DNA microarray membrane. The PCR products were denatured at 95°C for 5 min just before hybridization and chilled on ice for 2 to 5 min. Then PCR products were mixed with hybridization solutions and introduced into sample wells for a duration of 5 to 10 minutes. After washing, the membrane was treated to block unreacted areas. The results were visualized using NBT/BCIP (nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) solution, with positive HPV genotypes indicated by clearly visible indigo dots.

Statistical Analysis

To evaluate the methylation levels per disease category, boxplots were computed from the $\log_2$-transformed Δ(Δ)Cq ratios of the markers. Differences in methylation levels between disease categories were assessed using the Kruskal–Wallis test followed by Dunn's post-hoc test. A logistic regression model was built for distinguishing between high-risk (HSIL and cervical cancer) and low-risk (normal and LSIL) samples by R software version 4.0.2 ("glm2" package, https://cran.r-project.org/web/packages/glm2/index.html). The model was visualized by receiver operating characteristic (ROC) curves by the "pROC" package (https://cran.r-project.org/web/packages/pROC/index.html) and assessed through the area under the curve (AUC). The Cq values ≤35 for ACTB and ≤50 for the target gene were included in subsequent analyses. All other statistical analyses were performed in IBM SPSS Statistics software version 24.0 (IBM Corp). All reported *P* values were 2-sided. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical Features of Enrolled Participants

Baseline characteristics of the included individuals are shown in Table 1. In total, 228 individuals were included and divided into four groups, including 114 healthy individuals (controls), 46 patients with LSIL, 21 patients with HSIL and 47 patients with cervical cancer. The average age was highest for patients with cervical cancer (50.3±10.4 years) and lowest for controls (33.7±8.4 years). HPV-positivity was seldom seen in normal individuals (5.3%), while was quite common in the other three types (86.0% for LSIL, 100% for both HSIL and cervical cancer). The predominant HPV genotype was HPV 16/18, accounting for respectively 29.7, 63.2 and 66.7% of all HPV-positive patients with LSIL, HSIL and cervical cancer. The sensitivity of the HPV test for all types in HSIL and cervical cancer group was 100% (Table 2), while the specificity was 94.7% for normal individuals and only 14.0% for LSIL group. In comparison, the test for HPV16/18 had a lower sensitivity but

Table 1 Baseline Data Statistics of the Individuals Included in the Study

Features	Normal	LSIL	HSIL	Cancer
Number	114	46	21	47
Average age	33.7±8.4	43.2±13.9	40.3±11.3	50.3±10.4
HPV (all 21 types)				
Positive	6	37	19	3
Negative	108	6	0	0
(Missing)	0	3	2	44
HPV (HPV16/18)				
Positive	1	11	12	2
Negative	113	32	7	1
(Missing)	0	3	2	44

Abbreviations: HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions.

Table 2 Sensitivity and Specificity of Different Tests in Different Groups

Types	Sensitivity			Specificity		
	High-Risk	HSIL	Cancer	Low-Risk	Normal	LSIL
HPV (all 21 types)	100%	100%	100%	72.6%	94.7%	14.0%
HPV (HPV16/18)	63.6%	63.2%	66.7%	92.4%	99.1%	74.4%
CADM1	62.7%	60.0%	63.8%	93.1%	90.4%	100.0%
JAM3	62.7%	60.0%	63.8%	98.1%	97.4%	100.0%
FAM19A4	72.1%	57.1%	78.7%	97.5%	97.4%	97.8%
PHACTR3	63.2%	33.3%	76.6%	99.4%	99.1%	100.0%
PRDM14	67.2%	55.0%	72.3%	92.5%	91.2%	95.7%
SST	82.4%	61.9%	91.5%	94.4%	93.9%	95.7%
ZIC1	85.3%	66.7%	93.6%	98.1%	98.2%	97.8%
SFRP4	55.2%	50.0%	57.4%	84.4%	81.6%	91.3%
EPB41L3	74.6%	50.0%	85.1%	100.0%	100.0%	100.0%
SOX1	82.4%	70.0%	83.0%	96.3%	100.0%	93.5%
PAX1	79.1%	71.4%	87.2%	98.1%	99.1%	89.1%
ZNF671	91.5%	69.2%	100.0%	92.4%	93.7%	66.7%
Model	89.6%	71.4%	97.9%	95.0%	95.6%	93.5%

Abbreviations: HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions.

a higher specificity (Table 2). These results suggested a high sensitivity of HPV in distinguishing HSIL and cervical cancer, while a low specificity in distinguishing LSIL and normal individuals.

DNA Methylation Levels in Different Disease Stages

To investigate the correlation between DNA methylation levels and disease stages, the present study detected DNA methylation level of each gene using multiplex qMSP assays in patients with different statuses (Figure 1). Methylation levels were close between LSIL and normal group, while significant higher methylation levels were found for all markers in cervical cancer or HSIL group compared with LSIL and normal group (Figure 2). These findings indicated that methylation levels of all 12 markers increased significantly with severity of disease.

Figure 1 DNA methylation levels of all methylation markers across all four histological types. Predicted probabilities per sample are colored in different degrees of red.

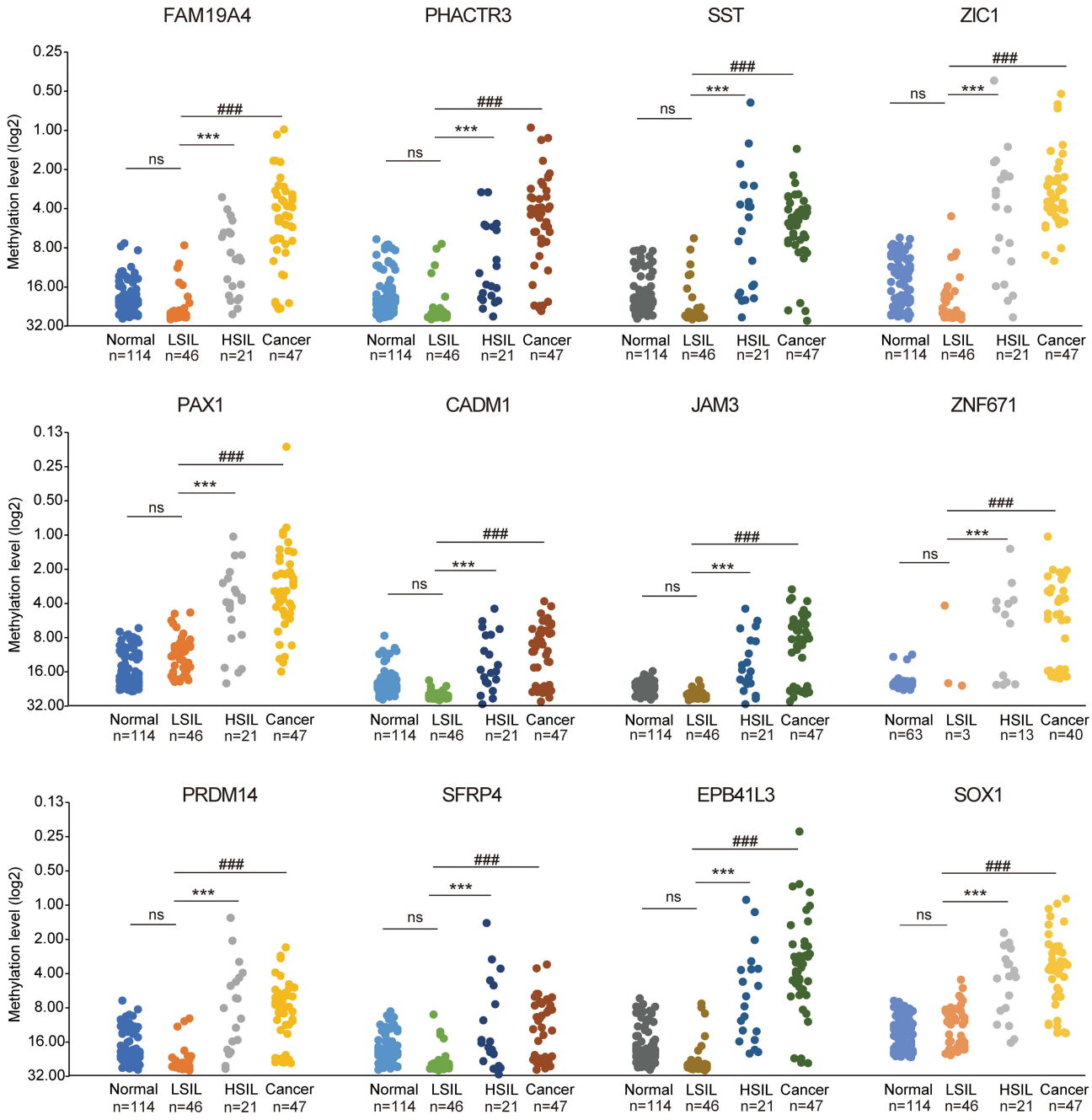

Figure 2 DNA methylation levels for the 4 types of categories for 12 markers. Differences between histological categories after the Kruskal–Wallis test, followed by the *t*-test or Mann–Whitney *U*-test. $^{ns}P>0.05$ in Normal group vs LSIL group; $^{***}P<0.001$ in LSIL group vs HSIL group; $^{\#\#\#}P<0.001$ in LSIL group vs Cancer group.

Diagnostic Performance of the 12 Methylation Markers

To compare the diagnostic performance of single methylation markers, all individuals were first divided into two groups: A high-risk group, including HSIL and cervical cancer patients; and a low-risk group, including normal and LSIL individuals. Marker-specific ROC curves demonstrated AUCs from 0.702 to 0.954 when discriminating between the high-risk group and the low-risk group (Figure 3A and B). The specificity and sensitivity of each gene in distinguishing individuals with different risks were summarized in Table 2. In detail, *ZNF671* had the highest sensitivity for the high-risk group (91.5%), with a relatively high specificity for the low-risk group (92.4%). *EPB41L3* had the highest specificity for the low-risk group (100%), with a quite low sensitivity for the high-risk group (74.6%). Notably, the specificity of

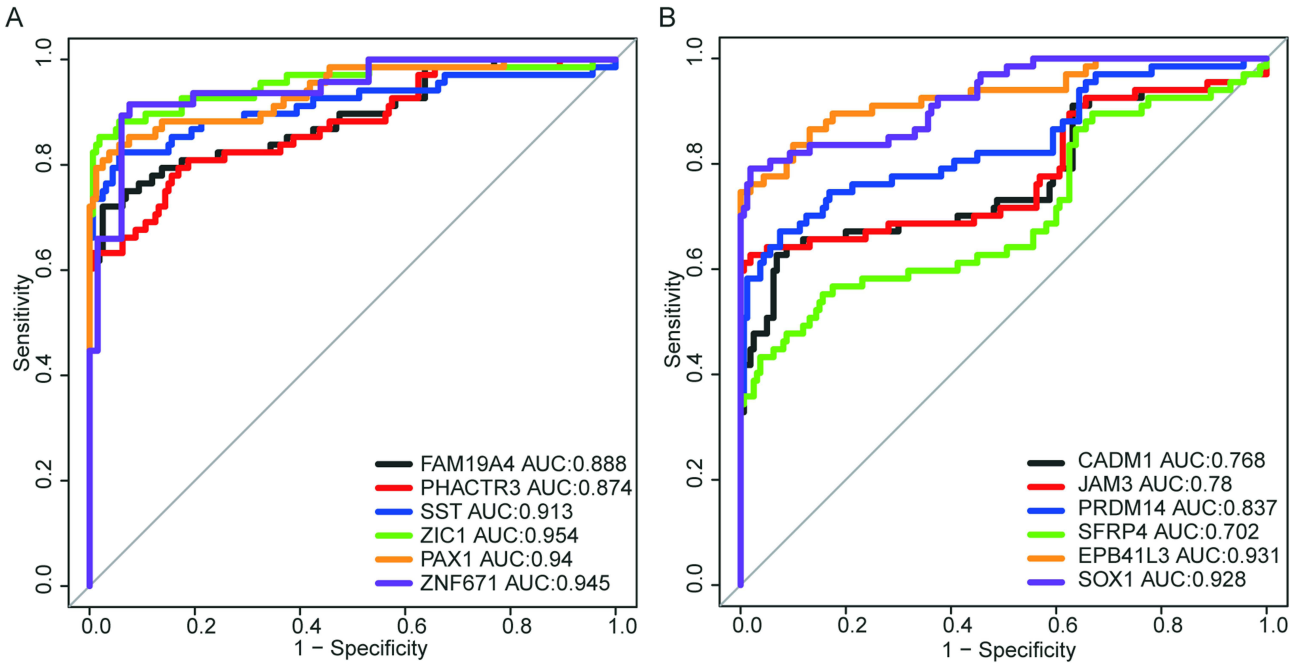

Figure 3 Diagnostic performance of the 12 markers in discriminating individuals with high-risk from individuals with low-risk. Univariate logistic regression analysis, ROC curves and AUCs of (**A**) FAM19A4, PHACTR3, SST, ZIC1, PAX1, ZNF671 and (**B**) CADM1, JAM3, PRDM14, SFRP4, EPB41L3 and SOX1.
Abbreviations: AUC, area under the curve; ROC, receiver operating characteristics.

these genes for detecting LSIL ranged from 84.4 to 100% and all of them were higher than the HPV test (14.0% in all types; 74.4% in HPV 16/18). These results suggested that the DNA methylation analysis could be combined with HPV testing as an early screening method for cervical cancer to avoid excessive medical treatment caused by false positives.

Constructing a Model for Predicting Disease Risk

To predict disease risk for each person more accurately through combinatorial screening, we combined data from six genes, including *FAM19A4, PHACTR3, SST, ZIC1, PAX1* and *ZNF671*, to build a best predictive model. Meanwhile, the methylation levels of these genes were validated by Sanger sequencing (Supplementary Figure S1). The calculation formula for the overall DNA methylation level (ODML) for each individual is $ODML=1.242958 - 0.001152 \times \Delta Cq_{FAM19A4} - 0.003311 \times \Delta Cq_{PHACTR3} - 0.009219 \times \Delta Cq_{SST} - 0.011352 \times \Delta Cq_{ZIC1} - 0.027571 \times \Delta Cq_{PAX1} - 0.009872 \times \Delta Cq_{ZNF671}$. The cutoff for this model was 0.451 (Figure 4). This model had a sensitivity of 89.6% and a specificity of 95.0% in distinguishing individuals in the high-risk and low-risk groups (Table 2). The AUC of this model was 0.969 and was higher than any AUC of single genes. Notably, compared with single genes, this model had the highest sensitivity (71.4%) in forecasting patients with HSIL.

Discussion

Although the HPV test has several advantages in cervical screening, it lacks specificity since most women infected with HPV will clear such an infection without developing lesions.[9] In clinical practice, clinical intervention is not required for women with a histopathological diagnosis of LSIL. However, HPV-positive women usually undergo colposcopy cervical biopsy, which could lead to cause damage to the cervix.[23] In the present study, only 14.0% of patients with LSIL were negative for HPV for all 21 types and 74.4% of patients with LSIL were negative for HPV test for type 16/18. The present result suggested that lots of women might undergo unnecessary colposcopy cervical biopsy. The low specificity of HPV tests could cause overtreatment and reduce patient compliance, therefore, new detection methods that can improve the accuracy of cervical cancer screening are needed.

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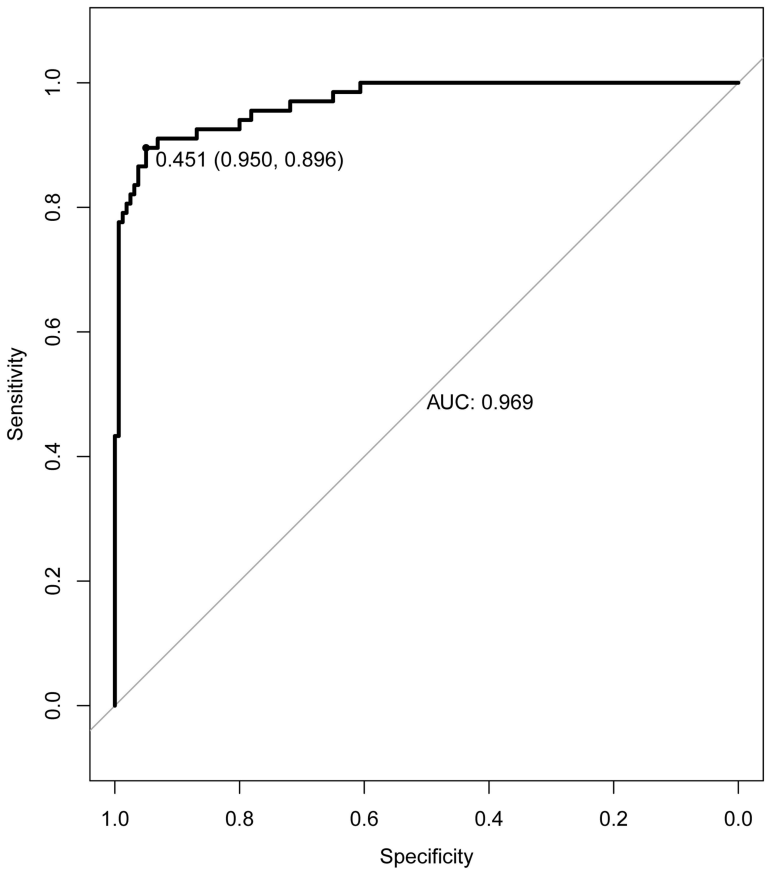

Figure 4 Diagnostic performance of predictive model combining six genes to distinguish individuals with high risk from individuals with low risk.

Methylation plays an important role in tumorigenesis and development and DNA methylation patterns were shown to be strongly associated with cancer phenotypes.[10,11] Currently, more than 100 methylation markers have been identified in cervical tissues. Among these markers, approximately 20 genes exhibit increased methylation levels in HSIL and cervical cancer, including *SST*,[12] *ZNF671*,[13] Ezrin,[14] *PAX1*,[15] *FAM19A4*,[24] *PHACTR3*,[25] *ZIC1*,[26] *CADM1*,[27] *JAM3*,[28] *PRDM14*,[25] *SFRP4*,[29] *EPB41L3*,[30] *SOX1* and POU4F3,[16] 12 of which were reconfirmed in the present study. The *PAX1* gene plays an important role in the growth and development of bone, spine, thymus and parathyroid gland.[31,32] Several studies have reported the aberrant methylation of *PAX1* in a variety of solid tumors, including cervical cancer. For example, Lai et al[33] reported that the *PAX1* gene is significantly hypermethylated in cervical cancer tissues compared with normal cervical tissues and the methylation level correlated positively with the tumor grade. Liu et al[34] also reported that the methylation level of the *PAX1* gene was significantly higher in the invasive cervical cancer group than in normal individuals, and patients with LSIL or HSIL. *ZNF671* was found with a great performance in detecting CIN3+.[35] The study showed that using *ZNF671* methylation test instead of TCT as a single triage strategy or as a combined triage strategy with HPV16/18 genotyping has achieved comparable sensitivity but higher specificity for CIN3+ detection among the high-risk HPV women.[35] Similarly, *FAM19A4* is a specific biomarker of cancerous lesions of the cervix, and it has been found that the methylation level progressively increases with the extent of cervical lesions.[36] *PRDM14* and *PHACTR3* methylation was detectable in early and late passages of the HPV16/18 immortalized keratinocytes as well as 33% CIN3 lesions.[25] *SST* and *ZIC1* methylation levels were identified to be a significant increase with severity of disease, reaching high levels in advanced CIN2/3 and cervical squamous cell carcinoma.[12,26,27] Additionally, *CADM1*, *EPB41L3*, *JAM3* and *SOX1* also observed to enhanced methylation levels and frequency with increasing severity of the underlying lesion.[27,28] In the present study, it is also found significantly higher methylation levels of *CADM1*, *EPB41L3*, *FAM19A4*, *JAM3*, *PAX1*, *PHACTR3*, *PRDM14*, *SFRP4*, *SOX1*, *SST*, *ZIC1* and *ZNF671* in cervical cancer or HSIL group compared with LSIL and

normal group (Cancer vs LSIL, $P<0.001$; HSIL vs LSIL, $P<0.001$; LSIL vs Normal, $P>0.05$). The specificity of these genes for detecting LSIL ranged from 84.4–100% and was higher than the HPV test, which has been reported to have a specificity of approximately 30–40%.[37] These results suggested that the DNA methylation analysis could be combined with HPV testing as an early screening method for cervical cancer to avoid excessive medical treatment caused by false positives.

In recent years, the combined detection of gene methylation biomarkers in diagnostic methods for cervical precancerous lesions and cervical cancer has been gaining attention from an increasing number of researchers.[13,38–40] For example, methylation specific PCR assay (GynTect®) was used to detect CIN2+ and CIN3+ in histological cervical specimens with a specificity of 51.6% and 42.2%, and a sensitivity of 83.2% and 91.2%, respectively.[13] Additionally, another research conducted constructed a predictive model for HSIL and cervical cancer, which was based on DNA methylation of *ASTN1, DLX1, ITGA4, RXFP3, SOX17* and *ZNF671*.[39] The AUC of this model was 0.850 with a sensitivity and specificity of 85% and 85.4%, respectively.[39] To further improve the accuracy of the cervical screening, we combined the methylation data of six genes, including *FAM19A4, PHACTR3, SST, ZIC1, PAX1* and *ZNF671*, and built a predictive model, in which only *ZNF671* was included by existing studies.[13,39] Encouragingly, while the sensitivity of this model for detecting individuals in the high-risk group was slightly lower than ZNF671, the sensitivity of this model for detecting individuals with HSIL and specificity for detecting individuals in the low-risk group was improved. In detail, the sensitivity of *FAM19A4, PHACTR3, SST, ZIC1, PAX1* and *ZNF671* for detecting individuals with HSIL ranged from 63.2–91.5% and the sensitivity of the model was 89.6%. The specificity of *FAM19A4, PHACTR3, SST, ZIC1, PAX1* and *ZNF671* for detecting individuals in low-risk group ranged from 92.4–99.4%, while the specificity of the model was 95.0%. Compared with reported models, this prediction model had the highest accuracy (sensitivity of 89.6%, specificity of 95.0%, AUC of 0.969) for predicting cervical cancer risk and its precancerous stages in Chinese women.[13,39] More importantly, the high sensitivity (71.4%) for detecting individuals with HSIL reduces missed diagnoses for high-risk populations, and the high specificity (93.5–95.6%) for detecting individuals in the low-risk group could avoid overtreatment, which might reduce the damage of cervical biopsy to patients with low risk.[41] However, for further application of this model, a larger sample size is still needed for validation, which remains a limitation of this study.

Conclusion

The present research first confirmed the high methylation levels of 12 cervical cancer-related genes in Chinese women with HSIL or cervical cancer. The constructed predictive model, which combines six methylation markers, exhibits high sensitivity (89.6%) and specificity (95.0%) in distinguishing high-risk individuals with HSIL or cervical cancer from low-risk individuals (AUC of 0.969). Excitingly, this model outperforms other existing models and single markers in terms of diagnostic accuracy, which is the novelty of this study. Meanwhile, the combination test of multiple methylation markers provides a more reliable tool for comprehensive evaluation of cervical cancer screening. It is a potential guide for the diagnosis of HSIL or cervical cancer in Chinese women.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

The study was carried out following the Declaration of Helsinki and approved by the Ethics Committee of Shanghai First Maternity and Infant Hospital in China (approval no. KS21275). The Ethics Committee of Shanghai First Maternity and Infant Hospital also approved a waiver of informed consent for this project.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

Zhonghe Ke reports a patent 202310419890.9 pending to Suzhou Yuntai Biomedical Technology Co., Ltd., and Shanghai First Maternity and Infant Hospital. The authors report no other conflicts of interest in this work.

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