Methylation of *SOX1* and *PAX1* Are Risk Factors and Potential Biomarkers for Cervical Lesions

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Abstract

Background: The correlation between methylation of paired box gene 1 (*PAX1*) and sex determining region Y-box 1 (*SOX1*) with human papillomavirus (HPV) infection and the progression of cervical lesions is not well understood. This study aims to explore the potential value of *PAX1* and *SOX1* as diagnostic biomarkers for cervical diseases.

Methods: A total of 139 cervical biopsy tissue samples were obtained from the Department of Pathology, the Seventh Medical Center, Chinese PLA General Hospital from 2021 to 2023. The samples include 32 cases of chronic cervicitis (inflammation group), 30 cases of low-grade squamous intraepithelial lesions (LSIL group), 50 cases of high-grade squamous intraepithelial lesions (HSIL group), and 27 cases of cervical squamous cell carcinoma (CSCC group). DNA was extracted from paraffin-embedded tissues, and the levels of HPV infection and methylation of *PAX1* and *SOX1* were detected.

Results: The methylation index (M-index) of *PAX1* and *SOX1* in the HSIL and CSCC groups is significantly higher than in the inflammation group (both P < 0.0001), with no significant difference between the LSIL and inflammation groups. There is no significant difference in the positive *PAX1* and *SOX1* methylation rate with HPV infection and age. The positive rates of *PAX1* methylation in the inflammation, LSIL, HSIL, and CSCC groups were 3.13%, 10.00%, 44.00%, and 88.89%, respectively. The positive rates of *SOX1* methylation were 3.13%, 10.00%, 40.00%, and 77.78%, respectively, and increasing with the progression of cervical lesions (R^2 = 0.9189/R^2 = 0.9279, P < 0.0001/P < 0.0001). Comparing LSIL, HSIL, and CSCC with the inflammation group and using cervical biopsy pathology diagnosis as the gold standard, methylation of *PAX1* and *SOX1* is a risk factor for HSIL and CSCC, with odds ratio (OR) values significantly increasing as lesions progress. The sensitivity of *PAX1* and *SOX1* methylation to cervical lesions increases with the progression of the lesions.

Conclusions: Methylation of *SOX1* and *PAX1* is not associated with HPV infection. The positive rate of methylation for *SOX1* and *PAX1* is positively correlated with cervical lesions, which can serve as potential biomarkers for HSIL and CSCC. They are risk factors and potential screening indicators for HSIL and above cervical lesions.

Keywords: Cervical squamous cell carcinoma; Methylation; PAX1; SOX1; HPV

Introduction

Cervical cancer (CC) is one of the most common malignancies among women worldwide, with a high incidence and mortality rate. It ranks fourth among causes of cancer-related deaths in women, thereby remaining a significant threat to female health [1]. Persistent infection with high-risk human papillomavirus (HPV) has been identified as the primary cause of CC [2]. The traditional screening methods are Pap smears, HPV testing, and colposcopy. Recent studies have suggested the role of DNA methylation alterations in the progression of CC, indicating that DNA methylation can serve as an effective biomarker for CC screening [3]. Studies have also shown that DNA methylation can be used as an effective biomarker for the diagnosis of CC [4].

Methylation of DNA involves the addition of a methyl group to the cytosine nucleotide in DNA, under the influence of DNA methyltransferases (DNMTs), leading to the formation of 5-methylcytosine. More than half of the genes in the genomes of vertebrate contain short regions rich in CG dinucleotides, known as CpG islands (CGIs), which are the primary sites of methylation [5]. The *PAX* gene family, named after the paired box DNA-binding domain, plays a crucial role in tissue development and cell differentiation during embryogenesis [6]. The findings demonstrated that the expression of *SOX1* is associated with early embryogenesis, development of the central nervous system, and maintenance of neural stem cells [7]. *SOX1* and *PAX1* are tumor suppressor genes that regulate processes related to cell proliferation and invasion [8, 9], Their methylation occurs at a high frequency in CC and significantly increases as the disease progresses [10]. However, current studies are mostly focused on the detection of cervical exfoliated cells, with fewer investigations on the use of *PAX1* and *SOX1* methylation in cervical tissue for the diagnosis of CC. The purpose of this study is to explore the potential value of

Table 1. The Clinical Characteristics of the 139 Patients

Parameter	Chronic cervicitis	LSIL	HSIL	CSCC
Number of patients	32	30	50	27
Age				
≤ 39	12	15	29	6
40 - 59	14	10	19	18
≥ 60	6	5	2	3
HPV				
Positive	2	19	46	24
Negative	30	11	4	3

LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CSCC: cervical squamous cell carcinoma; HPV: human papillomavirus.

PAX1 and *SOX1* methylation as diagnostic biomarkers for CC.

Materials and Methods

Study population and data collection

A total of 139 cervical biopsy tissues were collected from the Seventh Medical Center of PLA General Hospital, including 32 cases of chronic cervicitis, 30 cases of low-grade squamous intraepithelial lesion (LSIL), 50 cases of high-grade squamous intraepithelial lesion (HSIL), and 27 cases of cervical squamous cell carcinoma (CSCC). The inclusion criteria are as follows: 1) female patients aged between 20 and 75 years; 2) patients who underwent cervical biopsy under colposcopic examination, with the biopsy slides reviewed and diagnosed by two pathologists; 3) exclusion of specimens from patients with other malignant tumors, immunodeficiency diseases, other cervical lesions, or those who were currently pregnant. The clinical characteristics of the patients are provided in Table 1.

HPV DNA testing

HPV DNA testing in paraffin-embedded tissue samples blocks containing ≥ 20% lesions were selected, and DNA from tissue samples was extracted using the formalin-fixed paraffin-embedded (FFPE) tissue extraction kit (AmoyDx, China). The concentration and purity of the DNA samples were evaluated using Nanodrop (Thermo Fisher Scientific). HPV genotyping was performed using an HPV test kit (Kapa Biosystems, China) that can identify 21 HPV genotypes, including 15 high-risk HPV DNAs (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68) and six low-risk HPV DNAs (types 6, 11, 42, 43, 44, and 81).

Methylation testing for *PAX1* and *SOX1*

After extracting DNA from tissue samples, bisulfite conversion was performed, and then the methylation status was assessed by amplifying the converted DNA using multiplex polymerase chain reaction (PCR). DNA was extracted from paraffin-embedded tissues (Aidlab Biotechnologies, nucleic acid extraction reagent) and converted through a chemical reaction with bisulfite, which transforms unmethylated cytosine into uracil sulfonate through a deamination reaction. Multiplex PCR amplification was utilized to determine the bisulfite-converted DNA, thus detecting the methylation of *SOX1*, *PAX1*, and the internal control gene *ACTB* (Kapa Pharmaceuticals, methylation testing kits for *SOX1* and *PAX1* genes). The positive cut-off value for *PAX1* was 38, and for *SOX1*, it was 38.6. The formula for the methylation index (M-index) is M-index = $10{,}000 \times 2^{-|\text{Ct value of target gene - Ct value of internal control gene}|}$ [11].

Statistical analyses

The M-index is presented as mean ± standard deviation. A comparison between two groups was performed using the two-independent sample *t*-test. Data analysis was conducted using SPSS statistical software (version 21.0), and GraphPad Prism 8 was used for data visualization and statistical plotting. P value < 0.05 was considered statistically significant.

Ethics approval

This study was approved by the Ethics Committee of the Seventh Medical Center of Chinese PLA General Hospital (approval number: 2022-181) and was conducted in compliance with the ethical standards of the responsible institution on human subjects as well as with the Helsinki Declaration.

Results

A total of 139 patients were enrolled in this study from 2020 to 2023. These patients were divided into four groups: 32 in the inflammation group, with an average age of 45.40 ± 15.32 years; 30 in the LSIL group, with an average age of 41.16 ± 11.68 years; 50 in the HSIL group, with an average age of 40.62 ± 9.86 years; and 27 in the CSCC group, with an average age of 53.37 ± 9.05 years. The results revealed no significant difference between the two groups in terms of age. All patients were diagnosed by clinical pathologists. Representative hematoxylin and eosin (H&E) stained images of cervical biopsy tissues are shown in Figure 1.

Analysis of PAX1 and SOX1 methylation indices in cervical lesion tissues

We analyzed the methylation indices of *PAX1* and *SOX1* in 139 cervical biopsy tissue samples. The results indicated minimal methylation in the inflammation group, with median and quartile values of 0 for both *PAX1* and *SOX1*. In the LSIL group, the methylation values of *PAX* were 0. Similarly, for *SOX1*, the median and quartile values were also 0. There was no signifi-
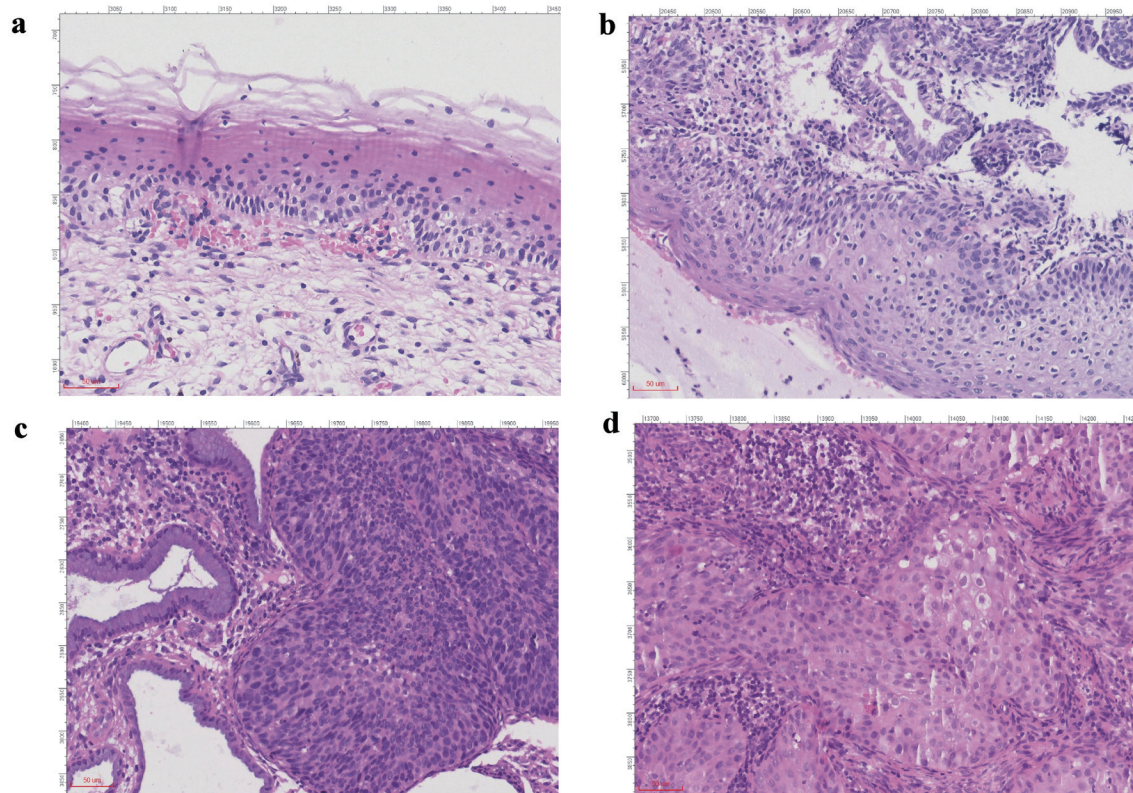
Figure 1. H&E staining images of patients in each group (× 100). (a) Inflammation group. (b) LSIL group. (c) HSIL group. (d) CSCC group. H&E: hematoxylin and eosin stain; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CSCC: cervical squamous cell carcinoma.

cant difference between the LSIL group and the inflammation group (P > 0.05) In the HSIL group, the methylation indices for *PAX1* and *SOX1* were 0.9150 and 36.21, showing a significant increase compared to the inflammation group (P < 0.0001). In the CSCC group, the methylation indices dramatically increased to 1,259 for *PAX1* and 1,743 for *SOX1*, which showed the highest values across all groups (Fig. 2, Table 2). These results indicate a significant increase in the M-Index of *PAX1* and *SOX1* in the HSIL and CSCC groups, highlighting a trend where the methylation levels increase progressively as cervical lesions advance.

The increased positive rate of *SOX1* and *PAX1* methylation during the progression of CC

We converted the quantitative Ct values into qualitative data and analyzed the methylation positive rates using 38 and 36 as the positive cut-off values for *PAX1* and *SOX1*, respectively. In the inflammation group, the positive rates for both *SOX1* and *PAX1* were 3.13%, with a concurrent positive rate for both *SOX1* and *PAX1* being 6.25%. In the LSIL group, the results showed that the positive rates for both *SOX1* and *PAX1* were 10%, with a concurrent positive rate of 13.33%. The positive rates for both *SOX1* and *PAX1* were 44% and 40% in the HSIL group, with a concurrent positive rate reaching 52%. In the CSCC group, the results showed that the positive rates for both

SOX1 and *PAX1* increased to 88.89% and 77.78%, respectively, with a concurrent positive rate reaching 88.89%. The research findings demonstrate that the positive rates of methylation for *PAX1* ($R^2 = 0.9189$), *SOX1* ($R^2 = 0.9279$), or both ($R^2 = 0.9367$) are positively associated with the progression of cervical lesions (P < 0.01) (Fig. 3a, Table 3). The trend is consistent in both single-gene (*PAX1* or *SOX1*) and dual-gene (*PAX1* and *SOX1*) analyses, with the positive rates for the dual-gene analysis slightly higher than those for the single-gene tests. Furthermore, the results of correlation analysis indicated that, within the diseased cervical tissues, there was no significant association between *PAX1*/*SOX1* methylation and either different age groups or HPV infection status (Fig. 3b, c, Table 3).

Evaluating the efficacy of HPV testing, *PAX1*, and *SOX1* methylation in identifying cervical lesions

We first evaluated the diagnostic efficacy of HPV for cervical diseases, and the results showed that HPV was identified as a risk factor for LSIL, HSIL, and CSCC (Table 4). Subsequently, we evaluated the efficacy of *PAX1* and *SOX1* methylation in identifying cervical lesions. In the LSIL group, the specificity of the methylation tests for both *PAX1* and *SOX1* genes is 96.88%, but the sensitivity and odds ratio (OR) are relatively low, being 10% and 3.44 respectively. The sensitivity of *PAX1* combined with *SOX1* is only 13.33%, with an OR of 2.31 (P =
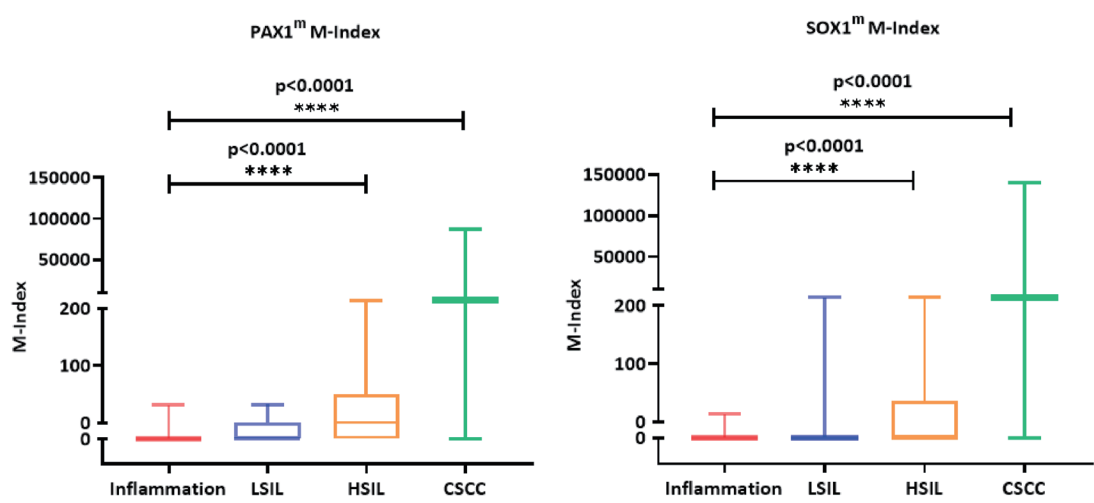
Figure 2. Methylation index of *PAX1* (a) and *SOX1* (b) in paraffin-embedded tissue samples of 139 patients undergoing cervical biopsy. LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CSCC: cervical squamous cell carcinoma; M-index: methylation index; *PAX1*m: *PAX1* methylation; *SOX1*m: *SOX1* methylation.

0.35, 95% confidence interval (CI): 0.39 - 13.64), suggesting that *PAX1* and *SOX1* methylation may not be significant risk factors for disease in the LSIL group. However, in the HSIL and CSCC groups, the sensitivity of the combined *PAX1* and *SOX1* gene methylation detection was 52.00% and 88.89%, respectively. The specificity of both groups was 93.75%, and ORs of 16.25 (P < 0.0001, 95% CI: 18.53 - 777.02) and 120.00 (P < 0.0001, 95% CI: 3.50 - 75.43) (Table 4). These results indicate that methylation testing of *PAX1* and *SOX1* genes has the potential to serve as a risk biomarker for patients with HSIL and higher grades of cervical lesions.

Discussion

CC is one of the most prevalent malignancies among females. Persistent infection by high-risk HPV types can lead to precancerous lesions or even CC. The combined testing of HPV and liquid-based cytology has been effective in preventing the occurrence and progression of CC [12]. However, traditional detection methods are subjective and lack precision, so there is an urgent need for more accurate methods. DNA methylation is an epigenetic modification and is considered a novel bio-

marker for the detection of CC. Previous studies have shown that methylation of the *PAX1* gene can be used as an auxiliary to routine cytology in CC screening [13]. Some studies have also confirmed the correlation between DNA methylation levels of genes such as *PAX1*, *SOX1*, *LMX1A*, and *ZNF582* and the occurrence and progression of CC [14, 15]. Nevertheless, the potential value of DNA methylation in CC screening or early diagnosis has not yet been fully elucidated. Large-scale studies are needed to further explore the potential methylation biomarkers for cervical lesions. *PAX1* is a tumor suppressor gene, and its high level of methylation is associated with the occurrence of tumors, which has been identified in cancers such as ovarian and oral cancers [16, 17]. The research results of Su et al suggest that *PAX1* can activate a variety of phosphatases, thereby regulating oncogenic kinases and exerting an effect on tumor growth and proliferation in cellular signal transduction [18]. The methylation of *SOX1* has been reported to be associated with various types of cancer, including liver cancer, lung cancer, and urothelial carcinoma [19-21]. In this study, by using the M-index as a measure, our results showed that the M-index for *PAX1* and *SOX1* was significantly higher in HSIL and CSCC than in the inflammation group, but there was no statistical difference in LSIL group compared to the

Table 2. Methylation Index of *PAX1* and *SOX1*

Group	*PAX1*		*SOX1*	
	M-index	**P**	**M-index**	**P**
Inflammation	0 (0, 0)		0 (0, 0)	
LSIL	0 (0, 0.45)	0.1095	0 (0, 0)	0.1718
HSIL	0.9150 (0, 50.01)	< 0.0001****	0 (0, 36.21)	< 0.0001****
CSCC	1,259 (514.7, 5,176)	< 0.0001****	1,743 (625, 3,711)	< 0.0001****

****P < 0.0001. M-index: methylation index; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CSCC: cervical squamous cell carcinoma.
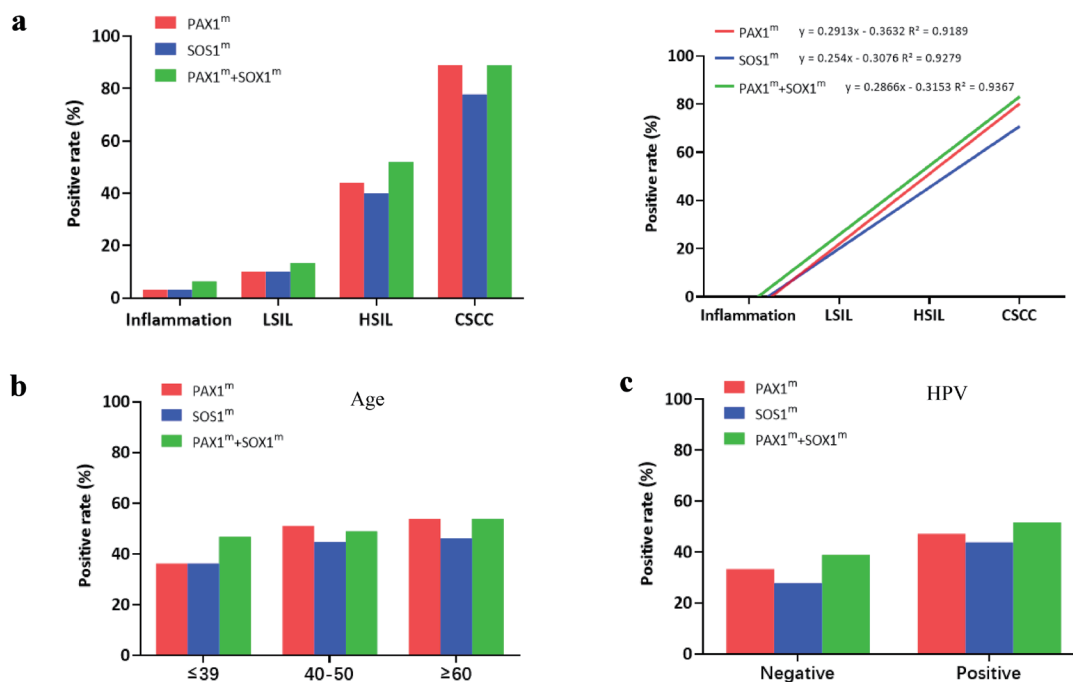
Figure 3. Methylation positivity rates of *PAX1* and *SOX1* in paraffin-embedded tissue samples of 139 patients undergoing cervical biopsy. (a) Methylation positivity rates of *PAX1* and *SOX1* in cervical biopsy tissue samples of patients in different stages. (b) Methylation positivity rates of *PAX1* and *SOX1* in cervical biopsy tissue samples of patients in different age groups. (c) Methylation positivity rates of *PAX1* and *SOX1* in cervical biopsy tissue samples of patients with HPV infection. LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CSCC: cervical squamous cell carcinoma; HPV: human papillomavirus; M-index: methylation index; *PAX1*m: *PAX1* methylation; *SOX1*m: *SOX1* methylation.

inflammation group. These findings suggest that *PAX1* and *SOX1* methylation may play an important role in the progression of cervical lesions to HSIL and CSCC stages.

According to literature reports, in samples of cervical exfoliated cells, high methylation of *PAX1* positively correlates with high HPV viral load [22]. However, it has been reported that gene methylation is associated with the progression of CC, but there is no significant correlation with HPV infection [23]. In this study, we assessed HPV in cervical tissue lesions to explore its correlation with methylation of *PAX1*/*SOX1*. Our findings suggest that HPV infection is not associated with *PAX1*/*SOX1* methylation. These results suggest that methylation of the *SOX1* and *PAX1* genes may be an independent factor in the development of CC. In addition, as the severity of cervical lesions increases, the positive rates of *PAX1* and *SOX1* methylation correspondingly increase, reaching the highest in CSCC. Zhang et al reported methylation positivity rates of *PAX1* and *SOX1* in LSIL at 43.7% and 19.6%, and in HSIL at 68.8% and 55.9%, respectively [10]. Huang et al noted the *SOX1* methylation rate exhibited a significant upward trend (P < 0.001), escalating from 27.27% in LSIL samples to 40.75% in HSIL samples, and culminating at 84.56% in CC samples [24]. Xu et al reported a methylation positivity rate of *PAX1* in LSIL samples at 9%, and in HSIL at 44% [25]. Compared to previous reports, our findings reveal discrepancies in *PAX1* and *SOX1* methylation positivity rates in LSIL but a higher consistency in HSIL and CSCC. These findings suggest that

the methylation of *SOX1* and *PAX1* may have a potential role in the later stages of cervical lesion development.

Currently, cytology and HPV testing have been widely used for CC screening. Patients diagnosed with HSIL require subsequent treatment, including ablation or cone biopsy [26]. However, about 10-24% of women diagnosed with HSIL who undergo loop electrosurgical excision procedure (LEEP) have only minor and mild lesions [27, 28], and this procedure is accompanied by complications including infertility, premature birth, and premature rupture of membranes. Therefore, for LSIL patients who are at risk of progressing to more serious diseases, more precise treatment strategies are needed. The p16/Ki-67 immunohistochemistry has emerged as a potential biomarker with high sensitivity and specificity for identifying HSIL [29]. However, this type of detection has a limitation in subjectivity. Therefore, exploring a reliable molecular biomarker for triage is particularly important. The results of a meta-analysis of seven studies involving 1,055 patients showed that *PAX1* methylation is associated with the transition from normal cervical epithelium to cervical intraepithelial neoplasia and cancer, which indicates its potential value as a biomarker for cervical diseases [30]. In the study, we revealed the potential value of *PAX1* and *SOX1* methylation in the diagnosis of cervical lesions, particularly in the identification of high-grade lesions. In the LSIL group, although methylation detection of *PAX1* and *SOX1* showed high specificity, the sensitivity and OR values were relatively low, indicating that

Table 3. Methylation Positivity Rates of *PAX1* and *SOX1* in Cervical Biopsy Tissue Samples of 139 Patients

	PAX1^m				*SOX1*^m				*PAX1*^m + *SOX1*^m			
	Negative	Positive	P	χ^2	Negative	Positive	P	χ^2	Negative	Positive	P	χ^2
Age												
≤39	30	17	0.27	2.59	30	17	0.65	0.86	25	22	0.9	0.21
	-63.83%	-36.17%			-63.83%	-36.17%			-53.19%	-46.81%		
40 - 59	23	24			26	21			24	23		
	-48.94%	-51.06%			-55.32%	-44.68%			-51.06%	-48.94%		
≥60	6	7			7	6			6	7		
	-46.15%	-53.85%			-53.85%	-46.15%			-46.15%	-53.85%		
HPV												
Negative	12	6	0.28	1.16	13	5	0.21	1.59	11	7	0.32	0.98
	-66.67%	-33.33%			-72.22%	-27.78%			-61.11%	-38.89%		
Positive	47	42			50	39			43	46		
	-52.81%	-47.19%			-56.18%	-43.82%			-48.31%	-51.69%		
Cervix												
Inflammation	31	1	< 0.0001	65.8	31	1	< 0.0001	50.7	30	2	< 0.0001	60.8
	-96.88%	-3.13%			-96.88%	-3.13%			-93.75%	-6.25%		
LSIL	27	3			27	3			26	4		
	-90.00%	-10.00%			-90.00%	-10.00%			-86.67%	-13.33%		
HSIL	28	22			30	20			24	26		
	-56.00%	-44.00%			-60.00%	-40.00%			-48.00%	-52.00%		
CSCC	3	24			6	21			3	24		
	-11.11%	-88.89%			-22.22%	-77.78%			-11.11%	-88.89%		

PAX1^m: *PAX1* methylation; *SOX1*^m: *SOX1* methylation; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CSCC: cervical squamous cell carcinoma; HPV: human papillomavirus; CI: confidence interval.

Table 4. Sensitivity, Specificity, and Odds Ratios (ORs) of HPV Detection, *PAX1* Methylation Detection, and *SOX1* Methylation Detection in 139 Patients

	Sensitivity	Specificity	OR	P	95% CI
Inflammation/LSIL					
HPV	63.33%	93.75%	25.91	< 0.0001	5.17 - 129.94
*PAX1*m	10.00%	96.88%	3.44	0.27	0.34 - 35.09
*SOX1*m	10.00%	96.88%	3.44	0.27	0.34 - 35.09
*PAX1*m + *SOX1*m	13.33%	93.75%	2.31	0.35	0.39 - 13.64
Inflammation/HSIL					
HPV	92.00%	93.75%	172.5	< 0.0001	29.72 - 1,001.24
*PAX1*m	44.00%	96.88%	24.36	< 0.0001	3.08 - 192.67
*SOX1*m	40.00%	96.88%	20.67	< 0.0001	2.61 - 163.80
*PAX1*m + *SOX1*m	52.00%	93.75%	16.25	< 0.0001	3.50 - 75.43
Inflammation/CSCC					
HPV	88.89%	93.75%	120	< 0.0001	18.53 - 777.02
*PAX1*m	88.89%	96.88%	248	< 0.0001	24.25 - 2,536.38
*SOX1*m	77.78%	96.88%	108.5	< 0.0001	12.16 - 967.80
*PAX1*m + *SOX1*m	88.89%	93.75%	120	< 0.0001	18.53 - 777.02

*PAX1*m: *PAX1* methylation; *SOX1*m: *SOX1* methylation; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CSCC: cervical squamous cell carcinoma; HPV: human papillomavirus; CI: confidence interval.

these methylation markers may not be sufficient as independent diagnostic tools in the early stages of cervical lesions. This finding is consistent with previous studies [31, 32], suggesting that at the LSIL stage, it may be necessary to combine other biomarkers or diagnostic methods to enhance the sensitivity of detection. However, in the HSIL and CSCC groups, methylation detection of *PAX1* and *SOX1* exhibited higher sensitivity and specificity, along with a significantly increased OR value, emphasizing the diagnostic value of these markers in the later stages of cervical lesions. These results highlight the potential value of *PAX1* and *SOX1* methylation as diagnostic biomarkers for CC, but its clinical applicability requires further investigation to validate its effectiveness. Our study also has certain limitations. Firstly, our study is limited to patients with cervical squamous cell carcinoma and does not include cervical adenocarcinoma, the effects of *PAX1* and *SOX1* methylation on cervical adenocarcinoma remain to be further validated. Additionally, our research sample size is limited, and more data and clinical follow-up are still needed for further exploration.

Conclusions

In conclusion, our study indicates that *PAX1* and *SOX1* methylation significantly increase HSIL and reach the highest levels in CSCC. As cervical lesions progress, the positive rates of *SOX1* and *PAX1* methylation gradually increase, showing good sensitivity and specificity for late-stage cervical lesions and serving as potential biomarkers for higher-grade cervical lesions. The findings of this study provide a theoretical foundation for the use of methylation markers in the clinical diagnosis of CC. However, further research is required to explore their broader application in clinical practice testing in the clinical diagnosis of CC.

Conflict of Interest

The authors declare no conflict of interest.

Informed Consent

Written informed consents were obtained from the patients for publication.

Author Contributions

YDL: writing - review and editing, writing - original draft, formal analysis, data curation. XYL: formal analysis, data cura-

tion. LWS: investigation, conceptualization, methodology. AJL: validation, supervision, conceptualization, writing - review and editing.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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