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ORIGINAL RESEARCH

A Sensitive and Simplified Classifier of Cervical Lesions Based on a Methylation-Specific PCR Assay: A Chinese Cohort Study

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Objective: The aim of this study is to assess the diagnostic and screening performance of a standardized methylation-specific real-time PCR assay targeting *SOX1* and *PAX1* genes for cervical cancer in a Chinese cohort.

Methods: Genomic DNA was extracted from cervical exfoliated cells and converted by sodium bisulfite and then analyzed by qMSP assay. Ct values were collected for *PAX1* and *SOX1* as target genes and *β-actin* as an endogenous reference gene. The samples included 295 cervicitis, 111 LSIL (low-grade squamous intraepithelial lesion), 51 HSIL (high-grade squamous intraepithelial lesion) and 30 cervical cancer.

Results: The Ct values decreased with the progression of cervical cancer from cervicitis, through LSIL and HSIL to cancer. The difference in Ct values between cytological grades was highly significant ($p \leq 0.01$) between grades either for *PAX1* or for *SOX1* except the difference between cervicitis and LSIL of *SOX1*. With the Ct cut-off values of *PAX1* gene and *SOX1* gene 38.6 and 38 and with the *PAX1*/*SOX1* in combination, the positive rate of methylation in invasive cancer tissues was 100%, in contrast to 11.5% (95% CI: 8.67%–14.33%) in cervicitis tissues, 45.1% (95% CI: 40.68%–49.52%) in LSIL tissues, and 68.5% (95% CI: 64.37%–72.63%) in HSIL tissues. The specificity and sensitivity of differentiating tumors from cervicitis were 0.957 (95% CI: 0.939–0.975) and 1.00, respectively. The specificity and sensitivity of differentiation between cervicitis+LSIL and HSIL+cervical cancer were 0.881 (95% CI: 0.852–0.91) and 0.748 (95% CI: 0.709–0.787), respectively.

Conclusion: *PAX1*/*SOX1* methylation could be translated into clinical practice for cervical neoplasia detection.

Keywords: Cervical cancer, DNA methylation, *PAX1*, *SOX1*, DMRs, qMSP detection

Introduction

Cervical cancer is among the most common gynecological malignant tumors in the world.[1] It ranks second only to breast cancer in female malignant tumors.[1] There are about 570,000 new cases and 311,000 deaths worldwide in 2015.[1] Scientific research has confirmed that persistent infection of high-risk papillomavirus (hrHPV) is the main cause of cervical cancer.[2,3] The development of invasive cervical cancers from the initial viral infections takes decades.[4] Only if a standard physical examination is regularly made, more than 90% of cervical cancer can be found and prevented.[5] Therefore, the selection of appropriate and effective diagnostic methods is of great significance for the prevention and treatment of cervical cancer.

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At present, the regular methods for cervical cancer screen are Pap smear, HPV DNA detection and colposcopy.[6] The successful promotion of Pap smear has greatly reduced the mortality and morbidity for patients with invasive cervical cancers in the past decades, but the accuracy of the tests varies greatly in different regions, depending on the performance of health care infrastructures and the experience of pathologists.[7] HPV DNA detection is highly sensitive but poorly specific.[7] Colposcopy is highly specific, but it requires a highly qualified operator.[7] For this reason, new biomarkers for cervical intraepithelial neoplasia2+ have been identified with great effort to improve risk stratification, and to distinguish women with benign lesion from those who require more intensive management.[8–10]

Epigenetic changes may play a part in the development of cervical cancer which indicates that DNA methylation may be useful as a marker for cervical cancer screening.[8–10] In 2008, Lai et al found six differentially methylated markers (*SOX1, PAX1, LMX1A, NKX6-1, WT1* and *OMECUT1*) related to cervical cancer through methylation chips.[11] Lim et al revealed that the methylation level of *PAX1* gene gradually increased with the progression of cervical lesions.[12] Sex determining region Y-box 1 (*SOX1*)[13] gene and Paired box 1 (*PAX1*) gene,[14] are tumor suppressors, which can inhibit cell proliferation and regulate the expression of invasion-related genes.[13] The genes *SOX1* and *PAX1* have been reported as potential methylation biomarkers and studies have demonstrated their promise in the detection of cervical intraepithelial neoplasms (CIN) grade 3 and worse lesions (CIN3+).[15–18] However, the published data were highly heterogeneous. No methylation markers can yet be utilized in cervical cancer screening or triage settings. Similar to other diagnostic molecular approaches, large, well-powered epidemiologic studies are still needed to identify and validate candidate methylation markers of cervical neoplasia. The key problems are: 1) whether the novel system is sensitive and specific enough to rival the traditional methods or become a complementary means of traditional cytological detection and HPV DNA detection; 2) whether the methylation detection system can be standardized and convenient for clinical application; 3) whether the novel system is population specific or regionally specific? All of these need to be tested by extensive and in-depth practice.

This study is intended to evaluate the diagnostic and screening performance of a methylation- specific real-time PCR assay, targeting the differentially methylated regions (DMRs) of *PAX1* and *SOX1* genes for cervical cancer in a Chinese cohort.

Materials and Methods

Patients and Clinical Specimens

During the period from October 2016 to December 2017, 487 specimens were collected from four medical centers in China, in order to assess the diagnostic and screening performance of the methylation detection kit ICervsure™. 487 specimens were divided into two groups: the case group and control group. The case group included 111 LSIL (low-grade squamous intraepithelial lesion), 51 HSIL (high-grade squamous intraepithelial lesion) and 30 CSCC (cervical squamous cell carcinoma). The control group included 295 cervicitis confirmed by cytological examination.

All the case group patients were diagnosed as LSIL, HSIL or CSCC by cervical biopsy under colposcopy. The control group patients were recruited from healthy women who received routine screening, and were diagnosed as cervicitis by histopathological examination. This study was approved by the Ethics Committee of Yunnan Tumor Hospital (the Third Affiliated Hospital of Kunming Medical University), Weifang Maternal and Child Health Hospital, Renmin Hospital of Wuhan University and Maternal and Child Health Hospital of Hubei Province (Women and Children's Hospital of Hubei Province) in China and was performed in accordance with the Helsinki Declaration. All participants provided written informed consent. All specimens were numbered and delinked from clinical information until data analysis.

The included specimens followed the criteria below: 1) women aged from 25 to 70 years, 2) women who had undergone cervical biopsy under colposcopy and diagnosed as LSIL, HSIL or CSCC and 3) women with clear results of histopathological examination and HPV test. The specimens with the following properties were excluded: patients having other malignant tumors, immunocompromised diseases, history of radio or chemotherapy, other cervical lesions, cervical surgery or current pregnancy.

Sampling Methods for Clinical Specimens

The specimens used in evaluation of the performance of the detection kit ICervsure™ were cervical exfoliated cells. Sampling was made as follows:

Patients took bladder lithotomy position, fully exposed the cervix, and removed from the excessive secretions. The tip of the sample brush was inserted into and pushed forward against the cervix. The brush was rotated in the same direction for 5 rounds. Immediately after sampling, the cervical exfoliated cells were put into the cell preservation solution and stored at 2–8° C for not more than 4 months. The cervical

exfoliated cell preservation solution was bought from Hubei Ruixinchang Biotechnology Co., Ltd. (Ehan Equipment 20150189).

DNA Extraction and Bisulfite Transformation

Genomic DNA was extracted from clinical specimens using the Nucleic Acid Extraction and Purification kit (Wuhan Ammunition Life Science and Technology Co., Ltd, Wuhan, China) according to the manufacturer's protocol. Sodium bisulfite treatment of genomic DNA from 1.4mL cell suspension was performed using the Bisulfite Conversion Kit (Wuhan Ammunition Life Science and Technology Co., Ltd, Wuhan, China) according to the manufacturer's manual. The bisulfited DNA was stored at −80°C until using.

qMSP Analysis with the Detection Kit ICervsure™

The Servsure™ is a methylation-specific detection kit developed by Wuhan Ammunition Life Science and Technology Co., Ltd. The testing information about the ICervsure kit can be found at http://www.ammulifetech.com/aigongshu.html, and information about the vendor at http://www.ammulifetech.com/. It is built on real-time multiplex polymerase chain reaction (qMSP) targeting the Sex determining region Y-box 1 (*SOX1*) gene and Paired box 1 (*PAX1*) gene. qMSP analysis was performed according to the manufacturer's instruction of the detection kit ICervsure™.

For the amplification reaction, 2 μL bisulfite-treated DNA (50 ng) was added in 25 μL amplification mix containing 1× Multiplex mix, 120nM of each primer (*β-actin, PAX1* and *SOX1*), and 120 nM of TaqMan probe of each target. Amplification and real-time measurement were performed in the 7500 ABI system (Applied Biosystems, Foster City, CA, USA), using the following conditions: 10 min at 95°C followed by 45 cycles of 20 s at 95°C and 45 cycles of 30 s at 60°C.

Test Results Analysis

After qMSP amplification, 3 Ct values of *PAX1, SOX1*, and *β-actin* were produced from each sample. Ct values of *PAX1, SOX1*, and *β-actin* and the results of cytological tests from the 487 specimens were summarized in Supplementary 1. Negative results showing no amplified signal were given Ct = 45. Testing results with Ct values of *β-actin*≥36 were defined as detection failure. For each sample, qMSP positivity was identified according to the Ct cut-off values (Ct=38 for *PAX1* and Ct=38.6 for *SOX1*). For each gene *PAX1* or *SOX1*, the sample was described as positive when its Ct value was lower than the cut-off value and as negative when its Ct value was higher than the cut-off value. For a combination of *PAX1* and *SOX1*, the sample was defined as positive only if any of these two genes showed a positive result.

The cut-off value of qMSP was determined by 10-time 10-fold cross-validation method. 487 clinical samples of cervical cancer (Supplementary Table 1) were split into 10 shares. Nine of them served as training data and one as testing data in turn. Correctness (or error rate) was obtained in each test. The accuracy (or error rate) data of 10 experiments were averaged to estimate the sensitivity of the algorithm. 10-fold cross-validation was repeated 10 times (10-fold cross-validation). The sensitivity value of each time was averaged as an estimate of the accuracy of the algorithm. According to the accuracy data obtained by 10-fold cross-validation, ROC curves and all associated statistics were generated. The optimal cut-off values were determined as maximizing the sum of sensitivity plus specificity.

On this basis, the positive rates of *PAX1* and *SOX1* methylation in specimens with different progression of disease were calculated.

Data Processing

SPSS 17.0 (IBM Corporation, Armonk, NY, USA) software was used for statistical analyses. Ct values obtained by qMSP were analyzed (Table 1) and plotted (Figure 1) according to clinical status using the exploration analysis of the description function. Two ended group-wise *t* test was used for the difference in the methylation level between different clinical states.

A "true positive" is the event that the test makes a positive prediction, and the subject has a positive result under the gold standard, and a "false positive" is the event that the test makes a positive prediction, and the subject has a negative result under the gold standard. Let A=true positive, B=false positive, C=true negative, D=false negative, then we have Sensitivity=A/(A+C)×100%, Specificity=D/(D+B) ×100%, and the Positive Predictive Value (PPV) =A/(A+B) × 100%.

Results and Analysis

Distribution of Ct Values of DMRs Was Closely Related to the Disease Progression of Cervical Cancer

According to cytological examination, 295 out of 487 patients were diagnosed with cervicitis, 51 with LSIL,

Table 1 Statistical Features of Ct Values of DMR Genes *PAX1, SOX1* and the Endogenous Gene *β-Actin*

		PAX1				*SOX1*				*β-actin*			
		Cervicitis	LSIL	HSIL	Cancer	Cervicitis	LSIL	HSIL	Cancer	Cervicitis	LSIL	HSIL	Cancer
Mean		43.43	39.74	36.90	30.54	44.37	42.65	37.99	29.87	26.84	26.48	26.41	26.35
95% Confidence	Lower	43.10	38.46	35.92	29.66	44.10	41.31	36.78	28.65	26.71	26.21	26.26	25.97
Interval	Upper	43.77	41.02	37.88	31.42	44.64	44.00	39.19	31.08	26.98	26.74	26.57	26.73
5% trimming mean		43.82	39.93	36.86	30.29	44.85	43.19	38.10	29.44	26.78	26.48	26.41	26.30
Median		45.00	38.99	35.58	29.86	45.00	45.00	35.37	29.23	26.64	26.46	26.39	26.32
Standard deviation		2.93	4.59	5.23	2.36	2.34	4.84	6.46	3.25	1.19	0.95	0.83	1.02
Minimum		31.66	28.99	28.49	27.99	29.86	28.63	27.22	26.58	24.12	23.72	24.39	24.44
Maximum		45.00	45.00	45.00	37.69	45.00	45.00	45.00	45.00	31.95	29.59	28.91	29.12
Range		13.34	16.01	16.51	9.70	15.14	16.37	17.78	18.42	7.83	5.87	4.52	4.68
Quartile distance		2.65	9.19	10.13	2.78	0.00	0.00	13.31	2.94	1.57	1.21	0.97	1.28
Skewness		−1.83	−0.25	0.47	1.68	−3.95	−1.75	0.04	3.65	0.93	0.19	0.11	0.76
Kurtosis		2.45	−1.08	−1.22	3.27	15.41	1.39	−1.81	16.85	1.61	2.25	0.58	1.00

111 with HSIL and 30 with CC. In this study, 487 specimens were tested with the methylation detection kit ICervsure™. The Ct values of *PAX1*, SOX2 and *β-actin* were produced for each specimen and analyzed by the exploratory analysis using SPSS software (Figure 1).

Figure 1 displays the exploratory data analysis of Ct values of the DMRs (*PAX1, SOX1*) in 487 patients. Each cytological grade composes of three box plots, representing the distribution of the Ct value of DMRs (*PAX1* and *SOX1*) and the endogenous reference gene *β-actin*, respectively. The extremes of the boxes define the upper and lower quartiles and the centre lines define the median. Whiskers indicate 1.5× interquartile range (IQR). Beyond IQR are defined as the outliers. The black point indicates the extraordinary value which is 1.5 times the box height away from the upper or lower edge of the box. The asterisk * represents the extreme value, which is three times the box height away from the upper or lower edge of the box.

No matter what grade of the tissue (either cervicitis, LSIL, HSIL or cancer), the Ct value of endogenous reference gene *β-actin* varies slightly from 26.3±1.0 to 26.8 ±1.2, indicating that Ct value of *β-actin* gene was very consistent and no relationship with the progression of cervical cancer, and that the DNA quality and quantity of the detection system are well controlled. Ct values of precancerous lesion samples (LSIL and HSIL) vary greatly, while the Ct values of cancer tissues are relatively concentrated. The Ct value of *PAX1* ranged from Ct=45 of cervical inflammation to Ct=29.9 of cancer. Similarly, Ct value of DMR_{SOX1} ranged from Ct=45 of cervical inflammation to Ct=29.2 of cancer.

Table 1 indicates the statistical features of Ct values of DMR genes *PAX1, SOX1* and the endogenous gene *β-actin*. Mean Ct values of *PAX1* were 43.4,39.7,36.9 and 30.5 for cervicitis, LSIL, HSIL and cancer, respectively, indicating that Ct values decreased with the progression of cervical cancer. The same trend was demonstrated for *SOX1*. It is worth noticing that the variation of Ct value in LSIL (SD=4.6 for *PAX1*, SD=4.8 for *SOX1*) and HSIL (SD=5.2 for *PAX1*, SD=6.5 for *SOX1*) is significantly larger than cervicitis (SD=2.9 for *PAX1*, SD=2.3 for *SOX1*) and cancer (SD=2.4 for *PAX1*, SD=1.0 for *SOX1*).

Ct values of different grades of the specimens were compared by *t* test. The difference in Ct value between cytological grades was highly significant ($p \leq 0.01$) between grades either for *PAX1* or for *SOX1* except the difference between cervicitis and LSIL of *SOX1*. It is indicated that there is a tendency that the more severe the disease the lower the Ct value, suggesting that the Ct value may become an indicator of cancer progression.

A Ct Cut-off Value Clearly Distinguishes Cancer from Non-Cancer Specimens

In order to evaluate the performance of the detection kit ICervsure™ in diagnosis of cervical cancer, ROC curve analysis was carried out (Figure 2). The specificity, sensitivity and AUC values are summarized in Table 2.

For cancer versus cervicitis, the specificity and sensitivity are 0.936 (95% CI: 0.914–0.958) and 1.00, respectively, for the *PAX1* at the Ct cut-off value of 37.82; 0.996 (95% CI: 0.99–1.00) and 0.963 (95% CI: 0.946–0.980) for *SOX1* at the Ct cut-off value of 32.20; 0.996 (95% CI: 0.99–1.00) and 1.00 for the combination of *PAX1* and *SOX1* at Ct cut-off value 31.93.

For HSIL+cancer versus cervicitis+LSIL, the specificity and sensitivity are 0.90 (95% CI: 0.873–0.927) and 0.75

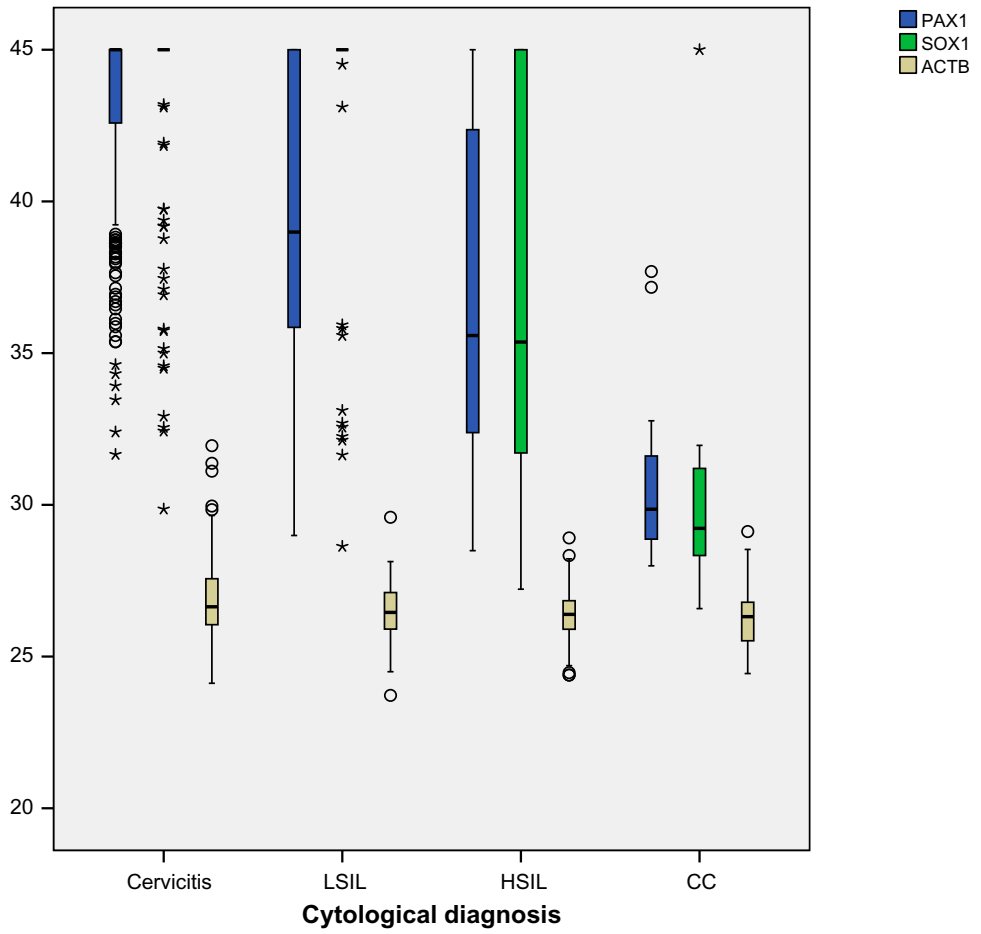

Figure 1 Exploratory data analysis of Ct values of the DMRs (PAX1, SOX1) in 487 patients. Each cytological grade composes of three box plots, representing the distribution of the Ct value of DMRs (PAX1 and SOX1) and the endogenous reference gene ACTB, respectively.

(95% CI: 0.712–0.788), respectively, for *PAX1*1 at the Ct cut-off value of 37.95; 0.926 (95% CI: 0.903–0.949) and 0.654 (95% CI: 0.612–0.696) for the *SOX1* at the Ct cut-off value of 38.58; 0.881 (95% CI: 0.852–0.910) and 0.748 (95% CI: 0.709–0.787) for the combination of *PAX1* and *SOX1* at the cut-off value of 37.92.

Considering all the situations as shown in Table 2, we decided to choose Ct=38.0 as the cut-off value of *PAX1* and Ct=38.6 as the cut-off value of *SOX1* in the qMSP detection of the clinical specimens of cervical cancer. That is, the specimen is considered methylation positive when the qMSP Ct≤38.0 for *PAX1* or qMSP Ct≤38.6 for *SOX1*. The specimen is considered methylation negative when the qMSP Ct≥38.0 for *PAX1* and qMSP Ct≥38.6 for *SOX1*.

Methylation Rate Increases with the Progression of Cervical Cancer

Based on the Ct cut-off value (Ct=38 for *PAX1* and 38.6 for *SOX1*), we were trying to transform quantitative Ct values into qualitative classification or positivity. Based on this classification, the positive rate was investigated for various grades of specimens. It was shown that the positive rate increased gradually with the disease progression regardless of *PAX1* or *SOX1* as the target. Results of qMSP detection were significantly positively correlated between *PAX1* and *SOX1* with the correlation coefficient r=0.8473.

As can be noted from Figure 3 that the probability of positive results increases with the progression of the disease. Single gene detection (*PAX1* or *SOX1*) showed the same trend as double genes in combination. The positive rate of *PAX1* was a little higher than that of *SOX1* in cervical cancer samples of any grade. The detection rate of double gene detection was slightly higher than that of a single gene.

The frequency of methylation at both the *PAX1* and *SOX1* increases with the increase of disease progression. For cancer samples, the methylation frequency of *PAX1* arrived at 100%. For the same grade samples, the

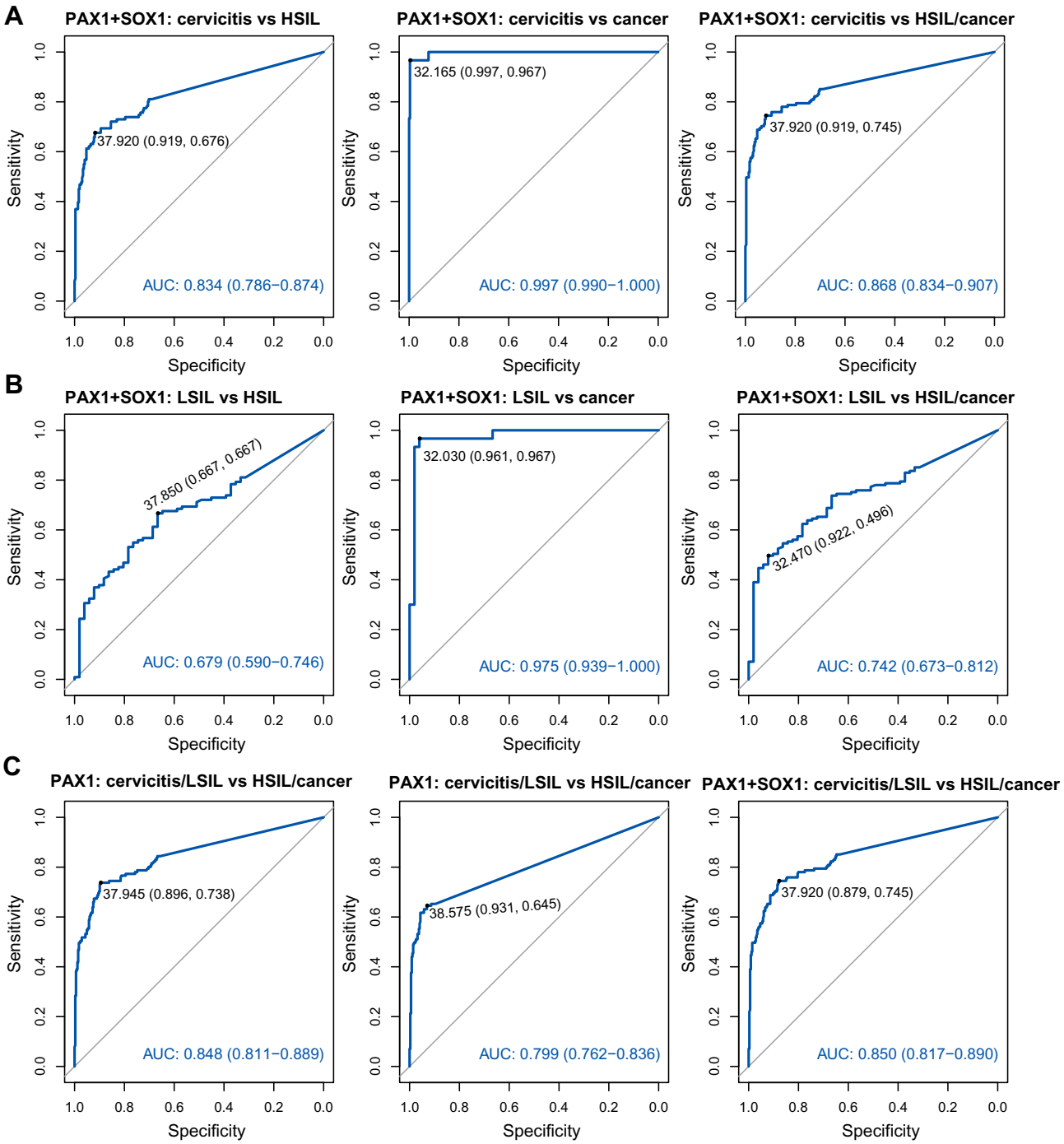

Figure 2 ROC curves of PAX1 and SOX1 assayed on scrapings of 487 patients (**A–C**).

methylation frequency of *PAX1* was consistently higher than that of *SOX1*. The methylation frequency of *PAX1* in LSIL samples amounted to 43.7% (95% CI: 39.3%-48.1%), while that of *SOX1* samples was only 19.6% (95% CI: 16.1%-23.1%). The methylation frequency of *PAX1* in HSIL samples was as high as 68.8% (95% CI: 64.7%-72.9%), while that of *SOX1* samples was only 55.9% (95% CI: 51.5%-60.3%).

Clinical Specificity and Sensitivity Allows Practical Utility for Early Detection of Cervical Cancer

When *PAX1* and *SOX1* genes were detected separately, the specificity and sensitivity of *PAX1* for cervical cancer were 0.934 (95% CI: 0.911–0.956) and 1.00, respectively, and that of *SOX1* was 0.996 (95% CI: 0.990–1.00) and

Table 2 Performance of the qMSP System in Detection of Clinical Specimens of Cervical Cancer

DMR	Control	Case	Sample Number		Ct Cutpoint	Specificity	Sensitivity	AUC Value
			Control	Case				
PAX1	Cervicitis	HSIL	295	111	37.95	0.93	0.67	0.83
PAX1	Cervicitis	CANCER	295	30	37.82	0.94	1.00	1.00
PAX1	Cervicitis	HSIL+CANCER	295	141	37.97	0.93	0.75	0.88
PAX1	LSIL	HSIL	51	111	37.85	0.72	0.85	0.68
PAX1	LSIL	CANCER	51	30	32.25	0.98	0.89	0.97
PAX1	LSIL	HSIL+CANCER	51	141	37.85	0.70	0.72	0.73
PAX1	Cervicitis+LSIL	HSIL+CANCER	346	141	37.95	0.90	0.75	0.86
SOX1	Cervicitis	HSIL	295	111	38.58	0.95	0.56	0.76
SOX1	Cervicitis	CANCER	295	30	32.20	1.00	0.96	0.98
SOX1	Cervicitis	HSIL+CANCER	295	141	38.58	0.95	0.65	0.81
SOX1	LSIL	HSIL	51	111	35.52	0.87	0.54	0.70
SOX1	LSIL	CANCER	51	30	32.03	0.96	1.00	0.98
SOX1	LSIL	HSIL+CANCER	51	141	35.52	0.87	0.63	0.76
SOX1	Cervicitis+LSIL	HSIL+CANCER	346	141	38.58	0.93	0.65	0.80
PAX1+SOX1	Cervicitis	HSIL	295	111	37.95	0.91	0.69	0.84
PAX1+SOX1	Cervicitis	CANCER	295	30	32.03	0.96	1.00	0.98
PAX1+SOX1	Cervicitis	HSIL+CANCER	295	141	35.52	0.87	0.63	0.76
PAX1+SOX1	Cervicitis+LSIL	HSIL+CANCER	346	141	37.92	0.88	0.75	0.85

0.963 (95% CI: 0.946–0.980). For the detection of LISIL and its above (LSIL, HSIL and CC), the positive coincidence rate, negative coincidence rate and total coincidence rate of *PAX 1* were 73.9%, 88.8%, 84.5%, and those of *SOX1* were 64.6%, 93.1% and 84.7%, respectively.

When *PAX1* and *SOX1* genes were detected in combination, the specificity and sensitivity of differentiating tumors from inflammation were 0.957 (95% CI: 0.939–0.975) and 1.00, respectively. The specificity and sensitivity of differentiation between inflammation + LSIL and HSIL + cervical cancer were 0.881 (95% CI: 0.852–0.709) and 0.748 (95% CI: 0.709–0.787), respectively.

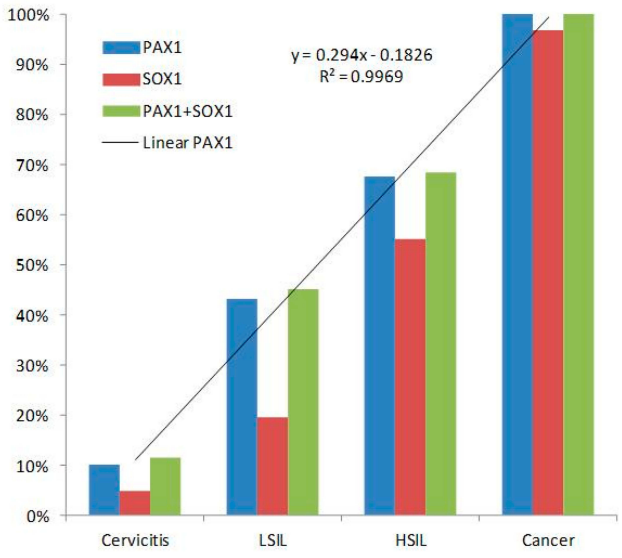

Figure 3 Detection rate of methylation among different cytological stages of cervical cancer.

Discussion

Cervical cancer is caused by human papillomavirus (HPV), which can be prevented and treated by HPV screening.[7] However, this virus infection is very common, and in most cases infected cervical cells do not turn into cervical cancer.[7] Therefore, positive HPV test results also need follow-up testing to the risk of each patient. Cytological examination is the primary shunt method at present, however, it lacks sensitivity and depends on experience. In these circumstances, ICervsure™ detection serves as a highly sensitive and specific tool for patients and doctors.

According to the meta analysis[15] of *PAX1* and *SOX1* methylation as an initial screening method for cervical cancer, the pooled sensitivity, and specificity for *PAX1* methylation test were 0.73 (95% CI: 0.70–0.75) and 0.87 (95% CI: 0.85–0.89), respectively, versus those of 0.71 (95% CI: 0.67–0.74) and 0.64 (95% CI: 0.61–0.67) for *SOX1* methylation. Paralleled *PAX1*/*SOX1* tests achieved AUC values of 0.89, under which, the pooled sensitivity was 0.72 (95% CI: 0.69–0.74); the pooled specificity was 0.77 (95% CI: 0.76–0.79), respectively. For the HPV DNA

testing, it yielded a pooled sensitivity of 0.81 (95% CI: 0.77–0.85) and specificity of 0.70 (95% CI: 0.67–0.72). With a combination of *PAX1* and *SOX1* in this study, the detection rates of LSIL, HSIL and cervical cancer were 46% (95% CI: 41.6%-50.4%), 67.6% (95% CI: 63.4%-71.8%) and 100%, respectively. The specificity and sensitivity to distinguish between cervicitis and cervical cancer were 100% and 95.7% (95% CI: 93.9%-97.5%). The performance of ICervsure™ has updated the best record of qMSP assay targeting *PAX1*/*SOX1* for cervical cancer and better than HPV DNA testing. In the present study, methylated CpG sites were recognized using specific primers and Taqman probes. Furthermore, the detection of fluorescence-labeled amplicons allows the sensitive and real-time measurement of the amplicons, thus enabling accurate quantification.

The positive predictive value of ICervsure™ was 107/152 = 70.39%, which is significantly higher than that of HPV (77/125 = 61.6%). Because most negative samples did not provide HPV test results, the positive predictive value of HPV in this experiment was considerably overestimated. Even this, ICervsure™ had significantly higher positive predictive value. Combined with HPV detection and ICervsure™ detection, the overall positive predictive value reached 81.9%. That is to say, positive predictive value of ICervsure™ is higher than HPV detection and the combination of ICervsure™ and HPV detection can improve the overall positive predictive value for cervical cancer detection. This is in accord with Tian et al[19] and Lorincz et al.[20] Tian et al also found that the dual methylated markers *PAX1*/ZNF582 resulted in a referral rate for colposcopic examination that was 24.4% of the rate for cytology.[20]

By now two reagent kits have been registered for cervical cancer detection worldwide. One is GynTect® produced by Oncgnostics company of German and another is QIAsure® by QIAGEN and Self-screen BV company. According to the product specifications for GynTect® available from https://www.oncgnostics.com/wp-content/uploads/2018/02/Eurogin-2017-talk.pdf and for QIAsure® available from http://www.egrinternational.com/QIAGEN/WomensHealth/QIAsure/PDF/Clinical-Data.pdf, the sensitivity of cancer detection is the same 100% for ICervsure™, GynTect and QIAsure, but ICervsure™ has the highest sensitivity of HSIL detection, being 76/111=68.5% (ICervsure™) > 53/80=66.3% (QIAsure®) > 30/49=61.2% (GynTect®). Sensitivity of HSIL + detection was 250/331=83.1% (QIAsure®) > 107/142=75.4% (ICervsure™) > 35/54=64.8% (GynTect. The specificity was 210/222=94.6% (QIAsure®) > 302/347=87.0% (ICervsure™) > 133/160=75.5% (GynTect). ICervsure™ possesses moderate and balanced sensitivity and specificity.

There have been numerous studies on use of DMRs for cancer diagnosis. Methylation index has been the most frequently used to evaluate the methylation level with qMSP. There are a variety of methods for calculating methylation index (MI), including $MI^{21}=\Delta Ct$, $MI^{22}=2^{-\Delta Ct}$ $MI^{17}=10{,}000\times 2^{\Delta Ct}$ where $\Delta Ct=Ct_{(Target)}-Ct_{(Endogenous\ reference)}$. $MI^{22}=2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$. $MI^{17}= Ct_{(Target)}/Ct_{(Endogenous\ reference)}$ and so on.[22,23] The cycle threshold (Ct) values for each target were normalized for DNA input. The values for all samples were transformed into relative quantity. However, we found that the amplification efficiency of *PAX1, SOX1* and *β-actin* was 1.04, 1.00 and 1.03, respectively and no significant difference between them. By strictly monitoring the initial sample size and controlling the Ct value of *β-actin* within a range of 26.5±1.2, the Ct value of DMRs becomes directly comparable. In this paper, we directly adopt Ct value to measure the methylation level and then convert the Ct value into methylation positivity according to the Ct cut point. This improvement greatly helps with the clinical application. The performance of PAX1 in combination with SOX1 seems not significantly better than that of the PAX1 single gene, although the combined test showed a slight improvement in precancerous detection (Figure 3). Further research is needed to determine whether the PAX1/SOX1 combination is superior to the PAX1 single gene.

Conclusion

PAX1 or *SOX1* methylation could potentially be treated as an auxiliary biomarker for cervical cancer screening. With ICervsure™ we provide a molecular test on the basis of epigenetic markers, which allows the triage of patients with an abnormal Pap smear and subsequent positive HPV test result. In contrast to other methods available in cervical cancer screening ICervsure™ will have the potential to decrease the high number of unnecessary cervical biopsies and surgeries.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

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